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(54) Title: PEGYLATION OF POLYPEPTIDES		
(57) Abstract <p>Compounds are disclosed having the general formula R_1-X-R_2, wherein R_1 and R_2 are biologically active groups, at least one of which is polypeptidic. X is a non-peptidic polymeric group. R_1 and R_2 may be the same or different. Preferred R_1 and R_2 groups are interleukin-1 receptor antagonist, 30kDa TNF inhibitor, interleukin-2 receptors and CR1 and muteins thereof. Also included are site selectively modified interleukin-1 receptor antagonist and 30kDa TNF inhibitor.</p>		

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PEGYLATION OF POLYPEPTIDESFIELD OF THE INVENTION

5 This invention relates to polypeptides that
have been covalently bonded to long chain polymers such
as methoxy polyethylene glycol. This invention also
describes methods and reagents for the reaction of
activated polymer molecules with various biologically-
10 important polypeptides.

BACKGROUND OF THE INVENTION

Many proteins that have been identified and
isolated from human and animal sources have been found
15 to show promising medicinal or therapeutic potential.
Great strides have been made in the methods for
identifying and characterizing such proteins, in
addition to methods for producing such proteins in
relatively pure forms and relatively large quantities.
20 As the development process advances in relation to the
utilization of such potentially valuable materials,
many obstacles have arisen in formulating these
compounds for use in clinical models.

For example, many such proteins have been found
25 to have an extremely short half life in the blood
serum. For the most part, proteins are cleared from
the serum through the kidneys. The systematic
introduction of relatively large quantities of
proteins, particularly those foreign to the human
30 system, can give rise to immunogenic reactions that,
among other problems, may lead to rapid removal of the
protein from the body through formation of immune
complexes. For other proteins, solubility and
aggregation problems have also hindered the optimal
35 formulation of the protein.

One of the most promising techniques for
addressing these problems has been covalently bonding
one or more inert polymer chains to the polypeptide of
interest. The most commonly used polymer is
40 polyethylene glycol (PEG), or monomethoxyl polyethylene

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glycol (mPEG). See, for example, Davis et al.,
Biomedical Polymers: Polymeric Materials and
Pharmaceuticals for Biomedical Use, pp. 441-451 (1980).
PEG is ideal for these purposes due to its proven non-
5 toxic properties. Other researchers have utilized
polyoxyethylated glycerol (POG) for similar purposes.
Knauf et al., J. of Biolog. Chem. vol. 263, pg. 15064
(1988).

Numerous results have been described whereby
10 the covalent modification of proteins with polyethylene
glycols ("pegylation") have resulted in the addition of
desirable characteristics to the protein. For example,
the pegylation of IL-2 has been shown to decrease the
clearance of IL-2 while not significantly affecting the
15 activity of the cytokine. The decreased clearance
leads to an increased efficiency over the non-pegylated
material. Katre et al., Proc. Natl. Acad. Sci. U.S.A.
vol. 84, pg. 1487 (1987).

Increasing the half-life of Superoxide
20 Dismutase (SOD) in blood serum has been a critical
barrier for the use of SOD for the treatment of various
symptoms. A number of studies have shown that the
pegylation of SOD will give rise to a decreased
clearance rate. See, for example, Conforti et al.,
25 Pharm. Research Commun. vol. 19, pg. 287 (1987).

Aggregation of Immunoglobulin G (IgG) has been
postulated as a factor that leads to serious side
effects to patients that are intravenously administered
IgG. It has been shown that the pegylation of IgG
30 reduces the aggregation of the proteins to prevent this
problem. Suzuki et al., Biochem. Biophys. Acta vol.
788, pg. 248 (1984).

The ability of pegylation techniques to affect
protein immunogenicity has also been shown. Abuchowski
35 and coworkers have studied the immunogenicity and
circulating life of pegylated Bovine Liver Catalase.
Abuchowski et al., J. Biol. Chem. vol. 252, pg. 3582

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(1977).

The addition of PEG groups to these various proteins decreases clearance due to the increase in molecular size of the pegylated protein. Up to a certain size, the rate of glomerular filtration of proteins is inversely proportional to the size of the protein. The ability of pegylation to decrease clearance, therefore, is generally not a function of how many PEG groups are attached to the protein, but the overall molecular weight of the altered protein. This has been borne out by clearance studies that varied both the size of the PEG side chains and the number of PEG units bonded to IL-2. Katre, supra.

The various studies of pegylated proteins in relation to clearance, immunogenicity, aggregation and physical properties all suggest that the PEG forms a flexible, hydrophilic shell around the protein. The PEG chains become highly hydrated and give the pegylated proteins a higher apparent molecular weight than would be predicted, and act to shield charges on the protein.

Because of the many promising results that have been seen in this field, a catalogue of procedures for the attachment of PEG units to polypeptides has been developed. The key element in these procedures is the "activation" of the terminal-OH group of the polyethylene glycol. Such activation is necessary in order to create a bond between the PEG group and the polypeptide. The vast majority of coupling procedures activate the PEG moiety in order to react with free primary amino groups of the polypeptides. Most of these free amines are found in the lysine amino acid residues.

In general practice, multiple PEG moieties are attached to the proteins. For example, in United States Patent No. 4,179,337 of Davis et al., it was found that to suppress immunogenicity it is desirable

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to use between 15 and 50 moles of polymer per mole of polypeptide.

Because multiple PEG chains are generally bonded to each polypeptide, and because there are typically a large number of lysine residues in each protein, there has been little effort to pegylate proteins to yield homogenous reaction products. See, Goodson et al. Biotechnology, vol. 8, pg. 343 (1990); U.S. Patent No. 4,904,584 of Shaw. This lack of reaction specificity gives rise to a number of complications. Among these, are that pegylation often results in a significant loss of activity of the protein. Presumably, attachment to a critical lysine residue could alter the active site of the protein rendering it inactive.

It has been shown in at least one system, that pegylation can lead to sterically hindered active sites. In other words, relatively small substrates may approach the protein, while the activity of proteins that react with larger substrates can be dramatically effected by random pegylation. Davis et al. supra. The site selective pegylation of such proteins could lead to modified materials that gain the desirable attributes of pegylation without the loss of activity. In addition, if the pegylated protein is intended for therapeutic use, the multiple species mixture that results from the use of non-specific pegylation leads to difficulties in the preparation of a product with reproducible and characterizable properties. This makes it extremely difficult to evaluate therapeutics and to establish efficacy and dosing information.

In certain cases, it has been found that the administration of multimeric complexes that contain more than one biologically active polypeptide or drug can lead to synergistic benefits. For example, a complex containing two identical binding polypeptides may have substantially increased affinity to the ligand

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or active site that it binds relative to the monomeric polypeptide. For this reason, multimeric complexes of proteins can be desirable in order to increase affinity of the protein to its ligand in addition to increasing the molecular weight of the complex.

Proteins frequently achieve their biological effects through interaction with other proteins. Where a simple complex of two proteins is sufficient to achieve the biological effect it has proved possible to mimic the physiological effects of endogenous proteins by administering exogenous proteins. However, where the biological effect requires the assembly of a complex containing more than two proteins it is more difficult to mimic the function of the endogenous proteins with recombinantly produced exogenous equivalents because the higher order complexes are frequently unstable. In such cases it may be advantageous to use crosslinked species containing two of the components of the complex to simulate the biologically-active complex.

Subsequent to the invention described herein, at least three research groups have described the production of crosslinked proteins, where the extracellular portions of one of the TNF receptors is attached to the heavy chain of human or mouse IgG, which are then crosslinked through disulfide bonds. Peppel et al., J. Exp. Med. vol. 174, pg. 1483 (1991); Ashkenazi et al., Proc. Natl. Acad. Sci. USA vol. 88, pg. 10535 (1991); and Loetscher et al., J. Biol. Chem. vol. 266, pg. 18324 (1991). In each case, the proteins were expressed in animal cell expression systems, and were found to be substantially more effective at inhibiting TNF than the monomeric soluble receptor alone. Similar procedures have also been used for producing similar crosslinked proteins of the CD4 protein, (Byrn et al., Nature (London) vol. 344, pg. 667 (1990)) the CR1 protein, (Kalli et al., J. Exp.

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Med. vol. 174, pg. 1451 (1991); Hebell et al., WO 91/16437 (1991)) and the CR2 protein. (Hebell et al., Science, vol. 254, pg. 102 (1991)).

5 These crosslinked proteins -- constructed of
two polypeptide units and a portion of the IgG antibody
-- have been shown to have promise as therapeutic
agents. The crosslinked proteins have an increased
molecular weight, which acts to decrease the rate of
clearance of the complex from the body, in addition to
10 the apparent enhancement of the affinity of the
proteins to their ligand. However, the proteins
crosslinked in this manner have so far only been
prepared by expression in animal cell expression
systems by the expression of fused genes. This has
15 been required in order to have the IgG portion of the
protein properly folded after expression. In addition,
the fixed heavy chain portion of the IgG antibody that
serves as the spacer or linker between the polypeptide
units does not allow for the ability to vary the
20 length, size or geometry of the spacer. Given the
apparent synergistic effect achieved by the dimeric
proteins, it is likely that by varying the spatial
orientation of the polypeptides the synergistic benefit
may be optimized. And finally, the crosslinked
25 proteins may be antigenic and/or have decreased
solubility. The heavy chain of antibodies is not
biologically inert.

Other dimeric or "bivalent" complexes have been
described. One such group of dimeric compounds has
30 been labeled hirulogs. These compounds are comprised
of very short polypeptide units that are linked by a
short poly-glycine spacer or linker. One of the
polypeptide units is a thrombin inhibitor -- a 5 amino
acid sequence taken from the 65 amino acid protein
35 Hirudin -- and the other is an anion-binding exocite
(ABE) recognition inhibitor. See, Maragonore et al.,
Biochemistry, vol. 29, pg. 7085 (1990); Bourdon et al.,

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FEBS vol. 294, pg. 163 (1991).

C-reactive protein (CRP) is an acute phase serum protein composed of five identical 23kDa subunits. CRP can induce reactions of precipitation and agglutination and can also react with Clg to activate the classical complement pathway. Cross linked oligomers of CRP have been formed using bis (sulphosuccinimidyl) suberate or 3,3'-dithio (sulphosuccinimidylpropionate) as cross-linking agents. Jiang et al., Immunology vol. 74, pg. 725 (1991).

The formation of dimeric or bivalent ligands for targeting opoid receptors has also been investigated. Non-peptidic β -naltrexamine or oxymorphone pharmacophores have been connected by short ethylene oxide or glycine spacers. Erez et al., J. Med. Chem. vol. 25, pg. 847 (1982); Portoghesi et al., J. Med. Chem. vol. 29, pg. 1855 (1986). Tetrapeptide enkephalins linked by short methylene bridges have also been designed to target opoid receptors, and have been shown to have a greater selectivity and affinity for the delta receptor than the original delta ligand. Shimohigashi et al., Nature vol. 197, pg. 333 (1982).

The cell surface glycoprotein CD4 has also been produced in multimeric forms through a sugar-based cross-linking strategy. The cross-linking agent utilized was bismaleimidoheptane (BMH). Chen et al., J. Biol. Chem. vol 266, pg. 18237 (1991).

Lymphocyte function-associated antigen-3 (LFA-3) is a widely distributed cell surface glycoprotein that is a ligand for the T lymphocyte CD2. LFA-3 with its associated lipids forms protein micelles of eight monomers which increased their ability to interact with cells with CD2 on their surface. Dustin et al., J. Exp. Med., vol. 169, pg. 503 (1989).

In a somewhat related technology, one group has studied the inhibitory effect of a synthetic

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polypeptide that is comprised of a repeating pentapeptidyl unit. The polymer was synthesized by the polymerization procedure with diphenyl phosphoryl azide to a size of about 10,000 daltons. The polymerized pentapeptide is one of the essential structures in several biological responses. Morata et al., Inst. J. Biol. Macromol. vol. 11, pg. 97 (1989).

A further obstacle in developing effective exogenous proteins to augment or compete with endogenous substances is that exogenous proteins must be administered systematically rather than being localized in the appropriate place. This can lead to lower efficacy and to increased side effects. Several groups have reported targeting bioactive proteins to the appropriate sites by linking them to other proteins that naturally home on those sites. Often such linkages are made through gene fusions between the active and the targeting proteins.

Polyethylene glycol spacer or linker units have been used to create antibody targeted superantigens after the date of the instant invention. A monoclonal antibody reactive to colon carcinoma cells was attached to the bacterial superantigen staphylococcal enterotoxin. Rather than being designed to exploit the benefits associated with the other bivalent complexes (e.g., higher molecular weight; synergistic effects of bivalency) these complexes are designed to target superantigens to specific locations. The pegylation process described to form these targeted superantigens creates a complex containing a large mixture of materials. The coupling of the antibody and the superantigen was accomplished by the use of N-succinimidyl 3-(2-pyridyldithio) proprionate and a 24-atom-long PEG-based hydrophilic space. According to this procedure 7 to 18 spacers were attached to each antibody unit and one or two lysines on each of the super antigens were reacted. Dohlsten et al., Proc.

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Natl. Acad. Sci. USA vol. 88, pg. 9287 (October, 1991).
Using this procedure it would be impossible to isolate
a single species in order to optimize the product or
process.

5 Two groups of proteinaceous materials having
significant applications for the treatment of a wide
variety of medical indications are Tumor Necrosis
Factor (TNF) inhibitors and Interleukin-1 receptor
10 antagonists (Il-1ra). These materials have been shown
to have beneficial effects in the treatment of TNF and
IL-1 mediated diseases respectively. Among the
indications that have been identified as being either
TNF mediated or IL-1 mediated, are Adult Respiratory
Distress Syndrome, Pulmonary Fibrosis, Rheumatoid
15 Arthritis, Inflammatory Bowel Disease and Septic Shock.

Copending U.S. Patent Application Serial No.
555,274, filed July 19, 1990, specifically incorporated
herein by reference, describes a class of naturally
occurring proteinaceous TNF inhibitors and a method for
20 manufacturing a substantial quantity of the same with a
high degree of purity. In particular, the
aforementioned application describes in detail two
subsets of TNF inhibitors referred to as 30kDa TNF
inhibitor and 40kDa TNF inhibitor. In addition to the
25 full-length 40 kDa TNF inhibitor protein, two
truncated, yet biologically-active, forms of the 40 kDa
TNF inhibitor have also been produced. These proteins,
in which 51 and 53 carboxyl termini amino acids have
been removed from the full-length protein, are referred
30 to respectively as 40 kDa TNF inhibitor Δ 51 and 40 kDa
TNF inhibitor Δ 53.

Copending U.S. patent application Serial No.
07/506,522, filed April 6, 1990, specifically
incorporated herein by reference, describes a preferred
35 class of naturally occurring, proteinaceous Il-1
inhibitors and a method for manufacturing a substantial
quantity of the same with a high degree of purity. In

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particular, the application describes in detail three such interleukin-1 inhibitors which are interleukin-1 receptor antagonists (IL-1ra's), namely, IL-1ra α , IL-1ra β , and IL-1rax.

5 Two additional classes of materials that are potentially useful for the treatment of a variety of medical indications are interleukin-2 inhibitors and complement inhibitors. Potential inhibitors of interleukin-2 include interleukin-2 receptors, the
10 extracellular portion of interleukin-2 receptors, interleukin-2 receptor antagonists, antibodies that recognize interleukin-2, and fragments of any of such species that contain the IL-2 binding function. Potential inhibitors of the complement system include
15 the receptor CR1, the extracellular portion of CR1, and the fragment of CR1 that contains the complement binding function.

Interleukin-2 receptor has been described and methods for its isolation have been disclosed in U.S. Patent No. 4,578,335 of Urdal et al. and U.S. Patent
20 No. 4,816,565 of Honjo et al. The gene encoding Interleukin-2 receptor and methods for its recombinant production have also been disclosed. European Patent Application No. 89104023.0 of Taniguchi et al.;
25 European Patent Application No. 90104246.6 of Taniguchi et al. See also, Honjo et al., Nature vol. 311, pg. 631 (1984); Taniguchi et al., Science vol. 244, pg. 551 (1989).

It could be assumed that to some extent the
30 soluble extracellular domain of either interleukin-2 receptor will act as an inhibitor to the action of the cytokine interleukin-2. Interleukin-2 is one of the best characterized cytokines, known to play a pivotal role in the antigen-specific clonal proliferation of T
35 lymphocytes. Interleukin-2 has also been shown to act on a variety of other cells in the immune system.

There are three discrete forms of the

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interleukin-2 receptor, comprised of two distinct receptor molecules designated either as IL-2 α and IL2r β .

5 The highest affinity IL-2 receptor is composed of two distinct IL-2 receptors. Both of these receptors have been cloned and characterized. The low affinity IL-2 receptor (IL-2 α) was cloned in 1984 and has been well characterized. Nikaido et al., Nature vol. 311, pg. 631 (1984). The extracellular domain of
10 the molecule has a molecular weight of 24,825 and has two N-linked glycosylation sites. The molecule contains 11 cysteines, 10 of which are involved in intramolecular disulfide bonds. The putative IL-2 binding domains on the molecule have been mapped both
15 by mutagenesis and epitope mapping. The intermediate affinity IL-2 receptor (IL-2r β) was cloned in 1989 and has not been as completely characterized as IL-2 α . Hatakayama et al., Science vol. 244, pg. 551 (1989). The extracellular domain of IL-2r β has a
20 molecular weight of 24,693. The molecule contains 8 cysteines and 4 N-linked glycosylation sites. The disulfide bonding in the molecule is unknown. IL-2r β has a cytoplasmic domain of 286 amino acids.

The disassociation constants (Kd's) for the IL-
25 2 receptors have been determined. They are 10⁻⁸M for IL-2 α , 10⁻⁹M for IL-2r β and 10⁻¹¹M for the high affinity receptor which consists of a complex of IL-2 α , IL-2r β and IL-2. Current models indicate that the formation of the high affinity complex is formed first by IL-2
30 binding to IL-2 α and then to IL-2r β . Ogura et al., Mol. Biol. Med. vol. 5, pg. 123 (1988).

An inhibitor of IL-2 may be valuable in the prevention of transplant rejection as well as autoimmune disorders. Currently, a monoclonal antibody
35 against IL-2 α that prevents IL-2 binding is being tested in human renal transplantation. Hiesse et al., La Presse Mediocle vol. 20, pg. 2036 (1991). In a

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study of 15 patients, the antibody, in combination with immunosuppressants, has been shown to be as effective in preventing allograft rejection as a control group getting higher doses of immunosuppressants. High
5 levels of circulating soluble IL-2 α have been detected in a number of diseases, some infections, as well as transplantation and rejection. This suggests involvement of IL-2 in these diseases.

CR1 is a protein also referred to as the
10 C3b/C4b receptor. CR1 is present on erythrocytes and a variety of other cell types, and specifically binds C3b, C4b, and iC3b. CR1 can also inhibit the classical and alternate pathway C3/C5 convertases and act as a cofactor for the cleavage of C3b and C4b by factor 1.
15 Fearon et al., Proc. Natl. Acad. Sci. USA vol. 75, pg. 5867 (1979). CR1 is a glycoprotein composed of a single polypeptide chain, and there are four allotypic forms. It is known that CR1 contains repetitive coding sequences, and this fact is used to explain the
20 existence of multiple allotypes. Krickstein et al. Complement vol. 2, pg. 44 (Abst.) (1985).

The diminished expression of CR1 on erythrocytes has been associated with systemic lupus erythematosus and CR1 number has also been found to correlate
25 inversely with serum level of immune complexes. The CR1 protein, the CR1 gene and methods for the production of CR1 are described in WO 91/05047 and WO 89/09220 of Fearon et al. As described above, dimeric species containing CR1 and portions of an antibody have
30 also been disclosed. WO 91/16437 of Hebell et al.

SUMMARY OF THE INVENTION

This invention relates to a method for modifying polypeptides and the resulting modified
35 polypeptides.

This invention includes substantially purified compounds comprised of the formula R_1-X-R_2 wherein R_1

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and R_2 are biologically active groups and X is a non-peptidic polymeric spacer. R_1 and R_2 may be the same or different groups, and at least one of the R_1 and R_2 is polypeptidic. In the preferred embodiments, R_1 and R_2 are selected from the group consisting of interleukin-1 receptor antagonist; 30kDa tumor necrosis factor inhibitor; interleukin-2 receptor and CR1, and X is selected from the group consisting of polyethylene glycol, polyoxyethylated glycerol, dextran, colonic acids, poly β -amino acids and carbohydrate polymers. Also included are pharmaceutical compositions comprised of such substantially purified compounds in a pharmaceutically acceptable carrier. Further included are methods of treating patients in need thereof with such pharmaceutical compositions. The compounds of the formula R_1 -X- R_2 , as depicted in Figure 19, are referred to as "dumbbells".

This invention also includes a method for the preparation of substantially purified therapeutically valuable compounds comprised of the formula R_1 -X- R_2 comprising reacting a non-peptidic polymeric group having at least two reactive groups capable of forming covalent bonds with the biologically active group R; and isolating said compound.

In an alternate embodiment, this invention includes a method for the preparation of substantially purified therapeutically valuable compounds, comprised of the formula R_1 -X- R_2 , wherein R_1 and R_2 are different, comprised of: reacting a non-peptidic polymeric group capable of forming covalent bonds when reacted with the biologically active group R_1 to form a complex R_1 -X; reacting complex R_1 -X with the biologically active group R_2 to form said compound; and isolating and purifying said compound.

In one embodiment, this invention relates to the site-specific pegylation of TNF inhibitor and IL-1 inhibitor proteins.

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In order to maintain site-specificity of pegylation, pegylating reagents are selected that will react almost exclusively with the free -SH groups of cysteine residues of the polypeptides. An example of a
5 pegylation reagent that covalently binds almost exclusively to the -SH groups of cysteine is O-(2-maleimido ethyl)-O' methlypolyethylene glycol.

Site specific pegylation may be done at either naturally occurring "free" cysteine residues of a given
10 polypeptide, or at free cysteines contained on muteins of the naturally-occurring polypeptides. Cysteines may either be added to or inserted into the amino acid sequence of the naturally occurring polypeptide, or substituted for other amino acid residues at selected
15 locations.

In one embodiment of this invention, the polypeptides that are to be pegylated are produced via recombinant DNA technology from a bacterial host cell. In most cases the bacterially expressed polypeptide
20 must be refolded to obtain biological activity prior to the pegylation step. In certain applications of this invention, the native polypeptide does not contain any free cysteine residues, but an altered polypeptide is produced to contain at least one free cysteine in the
25 biologically active polypeptide. According to this method, the refolding of the bacterially expressed polypeptide is facilitated by the addition, in turn, of a sulfhydryl containing compound such as cysteine and a disulfide containing compound such as cystine. After
30 refolding and purification, the polypeptide is treated with a limited amount of a mild reducing agent such as dithiothreitol ("DTT") to regenerate the sulfhydryl group of the novel cysteine residue of the altered polypeptide. Following dialysis under conditions
35 designed to prevent oxidation, the polypeptide may be reacted with a cysteine specific pegylation agent to site specifically form a covalently modified

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polypeptide.

Preferred pegylated polypeptides of the present invention are site-specifically pegylated TNF-inhibitors and IL-1 inhibitors. More specifically, this invention describes pegylated 30kDa TNF inhibitor and pegylated IL-1 receptor antagonist. Most preferred pegylated TNF inhibitors include 30kDa TNF inhibitor wherein the asparagine amino acid residue at position 105 of the native human protein is changed to cysteine using in vitro mutagenesis and pegylation has occurred at the free cysteine at position 105. Other pegylated derivatives of mutated 30kDa TNF inhibitors include mutations where cysteine has been added at positions 1, 14, 111 and 161. In addition to the singly pegylated muteins, any and all combinations of the various mutations may be included within a single mutein to create altered 30kDa TNF with more than one free cysteine residue capable of being pegylated.

The most preferred pegylated IL-1ra includes native or naturally occurring IL-1ra, which includes four free cysteines. Mono pegylation of the native IL-1ra yields site-specific pegylation at cysteine position 116. Other pegylated derivatives of mutated IL-1ra include muteins having cysteine added at the amino terminus of the polypeptide, cysteine added at positions 6, 8, 9, 84, or 141, and the replacement of the cysteine at position 116 with serine. In addition to the singly pegylated muteins, any and all combinations of the various mutations may be included to create altered IL-1ra with more than one free cysteine capable of being pegylated.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of the invention, including illustrative examples of the practice of the invention.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the amino acid sequence of native IL-1ra.

5 Figure 2 depicts the amino acid sequence of native 30kDa TNF inhibitor.

Figure 3 shows the Coomassie SDS-PAGE of unpegylated and pegylated forms of IL-1ra and the mutein c84s116 IL-1ra. Lanes 2, 3, 5 and 6 contain pegylation reaction mixes. Lanes 1 and 4 are the
10 unmodified proteins:

Lane 1 - IL-1ra

Lane 2 - mPEG^{*}₅₀₀₀ IL-1ra

Lane 3 - mPEG^{*}₈₅₀₀ IL-1ra

Lane 4 - c84s116 IL-1ra

15 Lane 5 - mPEG^{*}₅₀₀₀ c84s116 IL-1ra

Lane 6 - mPEG^{*}₈₅₀₀ c84s116 IL-1ra

Figure 4 depicts the mono S ion exchange chromatography of: Chromatogram A, the pegylation reaction mixture of mPEG^{*}₅₀₀₀ IL-1ra, peak 1 is the modified and peak 2 is the unmodified IL-1ra; and
20 Chromatogram B, shows the purified mPEG^{*}₅₀₀₀ IL-1ra.

Figure 5 depicts a size exclusion chromatogram showing the elution profile of several size standards, and mPEG^{*}₈₅₀₀ IL-1ra (fraction 7) and IL-1ra (fraction
25 13).

Figure 6 depicts the reverse phase HPLC fractionation of tryptic digest of alkylated mPEG^{*}₅₀₀₀ IL-1ra reacted with tritiated iodoacetic acid to label free cysteines. Separation was performed on a Brownlee
30 C8 (2.1 x 220mm) column at ambient temperature and a flow rate of 1000 μ L/min with a linear gradient. Solvent A was 0.1% TFA in water and solvent B was 0.085% TFA in 80% acetonitrile and 20% H₂O.

Figure 7 depicts the reverse phase HPLC
35 fractionation of chymotryptic digest of peptide 18 in figure 6. Conditions were identical to those in Figure 6. Peptides 5 and 8 contained tritium counts and

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peptide 5 had the amino acid sequence LCTAMEADQPVSL. The cysteine was identified as the carboxymethylcysteine derivative. This cycle was the only one containing counts above background. The amino acid sequence of peptide 8 began with serine 103 of IL-1ra. Redigestion of this peptide with chymotrypsin permitted fractionation of all tritium counts from the peptide.

Figure 8 depicts the plasma IL-1ra concentration versus time profiles of mature IL-1ra, pegylated IL-1ra, and several pegylated muteins of IL-1ra.

Figure 9 shows the SDS-PAGE gel showing c105 30kDa TNF inhibitor and mPEG, and the separation of unreacted 30kDa TNF inhibitor from mPEG c105 30kDa TNF inhibitor by size exclusion chromatography.

Figure 10 shows a plot containing intravenous plasma IL-1ra concentration versus time curves for a large number of singly PEGylated IL-1ra species, doubly PEGylated IL-1ra species, and IL-1ra PEG dumbbell species.

Figure 11 shows a plot containing subcutaneous plasma IL-1ra concentration versus time curves for a number of IL-1ra species as in Figure 10.

Figure 12 shows a plot of plasma IL-6 levels versus time after the injection of mice with hrTNF.

Figure 13 compares IL-6 levels induced in mice by five ratios of c105 30kDa TNF inhibitor to TNF (A) and five ratios of c105 30kDa TNF inhibitor PEG₂₀₀₀db to TNF (B).

Figure 14 depicts plasma IL-6 levels induced in mice by TNF alone and one to one ratios of TNF to c105 30kDa TNF inhibitor PEG₃₅₀₀ and PEG_{10,000} dumbbells.

Figure 15 depicts percent neutrophils induced by varying ratios of TNF to c105 30kDa TNF inhibitor (A), c105 30kDa TNF inhibitor PEG₃₅₀₀db (B); c105 30kDa TNF inhibitor PEG_{10,000}db (C); and c105 30kDa TNF

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inhibitor PEG_{20,000}db (D).

Figure 16 shows a plot containing intravenous plasma 30kDa TNF inhibitor concentration versus time curves for native 30kDa TNF inhibitor, c105 30kDa TNF inhibitor PEG₈₅₀₀, and PEG_{10,000} and 30kDa TNF inhibitor PEG₃₅₀₀, PEG_{10,000} and PEG_{20,000} dumbbells.

Figure 17 shows a plot containing subcutaneous plasma 30kDa TNF inhibitor concentration versus time curves for a number of 30kDa TNF inhibitor species as in Figure 16.

Figure 18 depicts the solubility of 3 solutions of native IL-1ra and c84 IL-1ra PEG₈₅₀₀ by plotting O.D. 405 versus time.

Figure 19 depicts the basic structure of compounds of this invention having the general formula R₁-X-R₂ that are referred to as dumbbell compounds.

DETAILED DESCRIPTION OF THE INVENTION

This invention involves the selective modification of pharmaceutically useful polypeptides, in particular, Tumor Necrosis Factor ("TNF") inhibitors and interleukin-1 ("IL-1") inhibitors. More specifically this invention describes the selective modification of 30kDa TNF inhibitor and IL-1 receptor antagonist ("IL-1ra"). The selective modifications serve to both enhance the pharmacokinetic properties of the polypeptides as well as to provide homogenous compositions for human therapeutic use.

Additional polypeptides that may be selectively modified according to the procedures of this invention include interleukin-2 receptors ("IL-2r") and CR1. All references to interleukin-2 receptor shall be construed to include both α and β chains of IL-2r unless stated otherwise.

In the preferred embodiments of the invention the modified polypeptides and DNA sequences are human.

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However, to the extent that there is sufficient homology between animal DNA and peptide sequences to the human forms, they would be included within the scope of this invention.

5 In one embodiment, the method of modification of the present invention includes covalently bonding long chain polymers to the polypeptides of interest in a site specific manner. The selected polypeptides may be the native or naturally occurring polypeptides of
10 interest, or they may be biologically active muteins of the polypeptides that have been produced to enhance the modification process described herein. The method of the invention includes the selection, production and screening of desired muteins that will meet the
15 objectives of this invention. In other embodiments of this invention the method for modifying polypeptides requires merely that the modification be made so that the resulting product be available in substantially purified form as that term is defined herein.

20 In certain embodiments, the modified polypeptides of the present invention will be bonded to long chain polymers at specific positions of the amino acid sequence. The modified polypeptides of the present invention will retain a substantial portion of
25 their biological activity. In the preferred embodiments, the modified polypeptides will retain at least one tenth of the biological activity of the native polypeptide in a receptor binding assay. In a more preferred embodiment, the modified polypeptide
30 will retain at least one fifth of the biological activity of the native polypeptide, and in the most preferred embodiment at least one fourth of the activity will be retained. In addition, the modified polypeptide will serve to improve the pharmacokinetic
35 performance of the native polypeptide in at least one of the following areas:

- 1) increasing the apparent molecular weight

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of the native polypeptide and, hence, reducing the clearance rate following subcutaneous or systemic administration;

5 2) increasing the solubility of the native polypeptide in aqueous solutions; or

 3) reducing the antigenicity of the native polypeptide.

 In many embodiments of the invention, each of these objectives will be accomplished. In the preferred embodiments of the invention, the long chain polymer will be polyethylene glycol or monomethoxy polyethylene glycol. A polyethylene glycol unit will be referred to herein as PEG and a monomethoxy polyethylene glycol unit will be referred to as mPEG. The approximate molecular weight of the polymeric unit will be given in subscripts. For example, a monomethoxy polyethylene glycol unit of approximate molecular weight of 5,000 will be depicted as mPEG₅₀₀₀ or PEG₅₀₀₀. Other long chain polymers included within the scope of this invention are polypropylene glycol ("PPG"), polyoxyethylated glycerol ("POG"), dextran, colonic acids or other carbohydrate-based polymers and polymers of β -amino acids and biotin derivatives.

 In an alternate embodiment of the present invention, the long chain polymer unit is dihydroxy polyethylene glycol, or HO-(CH₂CH₂O)_n-H. When activated to bind covalently with polypeptides or other biologically active compounds as described below, the dihydroxy material will contain two reactive sites.

 In the preferred embodiments of the present invention the long chain polymer units are bonded to the polypeptide via covalent attachment to the sulfhydryl group (-SH) of a cysteine residue. To obtain selectivity of reaction and homologous reaction mixtures, it is useful to utilize functionalized polymer units that will react specifically with sulfhydryl groups. The functional or reactive group

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attached to the long chain polymer is referred to herein as the activating group. Activating groups include the maleimide group, sulfhydryl group, thiol, triflate, tresylate, aziridine, oxirane and 5-pyridyl.

5 The preferred activating groups are maleimides.

Activated dihydroxy polyethylene glycols, because of the physical separation between the ends of the polymeric chain, are nearly equally reactive at each end of the molecule. By appropriate selection of
10 reaction conditions and polypeptides, the activated dihydroxy polyethylene glycols--or any other multi-activated long chain polymer unit--will react with polypeptides to form "dumbbell" shaped complexes where two polypeptides are joined by a long chain polymeric
15 unit.

By utilizing the different rates of reaction that would be found between the activated polymeric linked group and different cysteine-containing polypeptides and by the kinetics of the reactions, it
20 is easily within the skill of those in the art to also produce dumbbell complexes where substantially purified compounds can be formed comprising two different polypeptide groups, or comprising a single polypeptide group and a different biologically active group.
25 Examples of such heterodumbbell compounds are given below.

The extent and availability for reaction of cysteines varies dramatically from polypeptide to polypeptide. Therefore, in the biologically-active
30 form many polypeptides do not have "free" cysteines, or cysteines not bound to another cysteine. In addition, the existence of "free" cysteines does not mean that cysteines are accessible for binding to reactive reagents. Since the modification usually occurs on the
35 active or three dimensionally folded polypeptide, little or no reaction will occur when a free cysteine is found within the "interior" of the folded structure.

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5 A further constraint when modifying polypeptides is the potential effect the modification may have on the active site of the polypeptide. The modification of a cysteine having a certain proximal relationship to the active site may effectively deactivate the polypeptide. Even when a great deal is known about the selected polypeptide, it is difficult, if not impossible, to accurately predict which cysteine residues may be effectively modified.

10 The same factors also exist when mutated polypeptides are produced that contain additional cysteine residues. When the polypeptide is recombinantly produced via bacterial expression, the non-native cysteines may interfere with the proper
15 refolding of the polypeptide. In addition, the cysteine must be accessible to the pegylating reagent, and the pegylated cysteine must not significantly interfere with the active site of the polypeptide.

20 The selection of potential sites within a given polypeptide for the introduction of a non-native cysteine can be influenced based on various sources of information. For example, glycosylation sites may be a good site for a mutation to include a free cysteine. To the extent that information is known about the
25 binding or active site of the polypeptide, that information can also be used to select potential muteins. The addition or substitution of a cysteine residue at the amino terminus or carboxyl terminus of the polypeptide is also a likely prospect because of
30 its location. And finally, the mutation of lysine residues to cysteine may be considered based on the assumption that lysines will generally be found on the surface of the biologically active polypeptide.

35 Although a variety of potential muteins can be selected for a given polypeptide that may meet the desired characteristics, it is only through the synthesis, pegylation and testing of such altered

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5 muteins that it will be known which will meet the objectives of the present invention. In light of this invention and the general skill and knowledge of those skilled in the art, such synthesis, pegylation and testing can be performed without undue experimentation. It should be noted, that even if the pegylation of a polypeptide acts to reduce the biological activity of a polypeptide to a certain extent, the improvement in the pharmacokinetic performance of the polypeptide may greatly increase the value of the native polypeptide in various therapeutic applications.

10 Upon selection of target muteins, the preferred method for the production of the muteins is by recombinantly expressing the gene coding for the mutein. Assuming that the gene coding for the native polypeptide is known, the altered gene may be created either by standard site specific mutagenesis procedures on the native gene, or by the construction of the altered gene by standard gene synthesis procedures. These techniques are well known to those of ordinary skill in the art.

25 The gene coding for the target mutein may be expressed in a variety of expression systems, including animal, insect and bacterial systems. To the extent that expression systems have been perfected for the expression of the native polypeptides, the same systems may be used for the target muteins. In the preferred embodiment of the present invention, the genes coding for the target muteins are produced by site specific mutagenesis of the native gene, and the gene encoding the mutein is expressed from a bacterial expression system. The gene encoding native IL-1ra and a method for expressing said gene in E. Coli is described in detail in United States Patent No. 5,075,222 of Hannum et al., issued December 24, 1991. The gene encoding native 30kDa TNF Inhibitor and a method for expressing said gene in E. Coli is described in detail in United

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States Patent Application Serial Number 07/555,274 filed July 19, 1990. Each of these applications is incorporated herein by this reference.

5 The muteins and pegylated materials of the present invention include allelic variations in the protein sequence (sequence variations due to natural variability from individual to individual) and substantially equivalent proteins. "Substantially equivalent," as used throughout the specification and
10 claims is defined to mean possessing a very high degree of amino acid residue homology (See generally, M. Dayhoff, Atlas of Protein Sequence and Structure, vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated
15 herein by references) as well as possessing comparable biological activity. Also included within the scope of this invention are muteins and pegylated polypeptides that are partially truncated versions of the native polypeptide.

20 In one preferred embodiment of the method of the present invention when the target mutein is produced via recombinant DNA technology in a bacterial expression system, the following steps are performed:

25 1) The gene coding for the target mutein is created by site directed mutagenesis of the gene coding for the native polypeptide;

2) The gene coding for the target mutein is expressed in a bacterial expression system;

30 3) The target mutein is isolated from the bacteria and purified;

4) The target mutein is refolded in the presence of cysteine or another sulphhydryl containing compound;

35 5) The refolded target mutein is isolated and purified;

6) The purified and refolded target mutein is treated with a mild reducing agent;

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7) The reaction mixture is dialyzed in the absence of oxygen; and

8) The dialyzed reaction mixture is treated with a long chain polymer containing an activating group.

5 In the preferred embodiment for the production of pegylated muteins of 30kDa TNF inhibitor, the mild reducing agent is dithiothreitol ("DTT"). In an alternate embodiment, the modification may occur prior to the refolding of the expressed protein or mutein.

10 In the preferred embodiment of the present invention, the pegylated muteins and pegylated native polypeptides may be purified and formulated into pharmaceutical compositions by conventional methods. In an alternate embodiment, the purified muteins may also be formulated into pharmaceutical compositions.

15 The pegylated polypeptides of the present invention formed by the reaction of a deactivated long chain polymer unit have additional beneficial properties. These "dumbbell" shaped molecules can contain two of the polypeptides of interest attached by a single polymer unit. This structure imposes a certain amount of linearity to the polymeric molecule and reduces some of the steric hinderance inherent in the use of large hydrophilic polymers such as polyethylene glycol. The goal of obtaining molecules with increased apparent molecular weight is achieved while retaining high biological activity. Included specifically within the scope of this invention are bidentate molecules where two IL-1ra molecules or two TNF inhibitor molecules are covalently attached to a single polymeric chain, or where two different polypeptides are attached to a single polymeric chain, i.e., a single bidentate molecule containing both a TNF inhibitor and a IL-1ra moiety.

30 35 Native IL-1ra (figure 1) and various muteins of IL-1ra have been pegylated according to the present

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invention. Pegylation of wild type IL-1ra at free
sulphydryl groups, by the methods described in the
examples below, results in the addition of mPEG at the
cysteine residue at position 116 of IL-1ra (c116). The
5 other three cysteines are not accessible for pegylation
in the fully native molecule. To attach mPEG molecules
at different sites of IL-1ra and to make mPEG
conjugates having more than one mPEG, IL-1ra in which
native amino acids in IL-1ra were replaced with a
10 cysteine, or additional cysteines are added at the
amino-terminus of the protein. To prepare conjugates
in which residue 116 is not pegylated c116 has been
changed to a serine in a number of the muteins. Below
is a list of the muteins that have been generated for
15 reaction with mPEG (the residue numbering is based on
the sequence given in Figure 1; c referring to cysteine
and s referring to serine):

	c0s116	c0c116
	c84s116	c84c116
20	c6s116	c6c116
	c8s116	c8c116
	c9s116	c9c116
	c141s116	c141c116

Native 30kDa TNF inhibitor (figure 2) does not
25 contain any free cysteine residues. The following
muteins of 30kDa TNF inhibitor have been prepared (the
residue numbering is based on the sequence given in
Figure 2; c referring to cysteine):

	c105 30kDa TNF inhibitor
30	c1 30kDa TNF inhibitor
	c14 30kDa TNF inhibitor
	c111 30kDa TNF inhibitor
	c161 30kDa TNF inhibitor

Included within the scope of this invention is
35 an entire class of compounds, as depicted in Figure 19,
that can be represented by the formula R_1-X-R_2 wherein
 R_1 and R_2 are biologically active groups and at least

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one of R_1 and R_2 is polypeptidic, and X is a non-peptidic polymeric spacer or linker group. R_1 and R_2 may be the same group or different. Where R_1 and R_2 are different groups, both R_1 and R_2 may be

5 polypeptidic, or R_1 may be polypeptidic and R_2 may be any biologically active group. The compounds having this structure, which have been referred to as "dumbbell" compounds, are characterized by being

10 substantially purified. "Substantially purified" in this context is defined as being a homogenous composition.

A homogenous composition consists of one molecule of the linker X and one molecule of R_1 and one molecule of R_2 . A homogenous composition includes, but

15 does not require, that the biologically active groups R_1 and R_2 be attached to the linker at the exact same location on the groups in each molecule of the compound. In certain embodiments of the invention, the biologically active groups are attached site

20 specifically to the linker. For example, in the compound c105 30kDa TNF inhibitor PEG₃₀₀₀db, two c105 30kDa TNF inhibitor groups are attached at the 105 cysteine residue to the PEG₃₀₀₀ linker.

When referring to a "homogenous composition" it

25 is to be understood that on a molecule-by-molecule basis, the dumbbell compound is also not necessarily homogenous with respect to the exact length of the spacer group. It is understood by those skilled in the art that any production process that utilizes a given

30 weight range of PEG or other higher molecular weight polymer begins with a solution that contains an "average" molecular weight. Therefore, when a bis-reactive PEG unit is reacted with a polypeptidic group, the PEG unit is by definition polydisperse, and the

35 resultant dumbbell compound is heterogenous to the extent that the length of the linker is subject to the variation known to exist by those skilled in the art.

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In summary, "substantially purified" in this context refers to materials that are substantially free from compounds: 1) that deviate in the composition of R_1 or R_2 ; or 2) that are linked together by more than one linker X.

R_1 and R_2 are defined as being biologically active groups. Biologically active groups include any compound that can induce a biological effect on interact with a natural biological molecule.

Biologically active groups include proteins, polypeptides, steroids, carbohydrates, organic species such as heparin, metal containing agents, vitamins, or any other biologically active species. At least one of the groups R_1 and R_2 is polypeptidic. In the preferred embodiment, both R_1 and R_2 are polypeptidic.

Polypeptidic is defined as any compound that is substantially proteinaceous in nature. However, a polypeptidic group may contain some non-peptidic elements. For example, glycosylated polypeptides or synthetically modified proteins are included within the definition.

The biologically active groups R_1 and R_2 include binding groups and targeting groups. Binding groups are defined by their affinity for a given biological ligand. Targeting groups are defined by their ability to direct the location of a complex within a biological system. R_1 and R_2 may have affinity for the same ligand, in which case the dumbbell may have enhanced affinity to that ligand. R_1 and R_2 may have an affinity for different ligands, wherein R_1 serves to target the complex into a location where the ligand for R_2 predominates.

Preferred polypeptidic groups are receptors, the extracellular portions of receptors, cell surface molecules, and extracellular matrix molecules, binding proteins, and receptor antagonists. Included among the polypeptidic groups that may be used as R_1 or R_2 are

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the following polypeptides and any fragment thereof:
IL-1 receptor antagonist, 30kDa TNF inhibitor, 40kDa
TNF inhibitor, IL-2 receptor, CR1 (all references to
CR1 include any single or combination of consensus
5 repeat sequences of CR1), PDGF receptor, IL-2, MCSF
receptor, EGF receptor, IL-5 receptor, IL-3 receptor,
GMCSF receptor, T-cell receptor, HLA-I, HLA-II, NGF
receptor, IgG (V_H , V_L), CD40, CD27, IL-6 receptor,
Integrins CR3, VLA₄, ICAM, and VCAM, CR2, GMP140 Lec
10 domain, Laminin binding protein, Laminin fragments,
Mannose binding protein, exon 6 peptide of PDGF, and
proteases (with 2 catalytic domains or a target domain
and a catalytic domain). All references to receptors
includes all forms of the receptor whenever more than a
15 single form exists. In the preferred embodiments, the
groups R_1 and R_2 are selected from the group consisting
of IL-1 receptor antagonist, 30kDa TNF inhibitor, CR1,
and IL-2 receptor (both the α and β chains).

In a preferred embodiment, the non-peptidic
20 polymeric spacer X may be further defined as follows:
 $X = -Y_1-(Z)_n-Y_2-$, wherein Y_1 and Y_2 represent the residue
of activating groups that react with R_1 and R_2 to link
the spacer to the groups R_1 and R_2 , and $(Z)_n$ represents
the base polymeric group. According to the present
25 invention n is greater than 6 and preferably is greater
than 10.

Non-peptidic is defined as a polymeric group
that is substantially not peptidic in nature. The
inclusion of less than 50% by weight of α -amino acid
30 residue as part of Y_1 , Y_2 and Z would be considered
substantially non-peptidic in nature and would be
considered non-peptidic. In the preferred embodiment,
the non-peptidic spacer X is non-immunogenic, and
biologically inert and hydrophilic. In addition, the
35 preferred linkers are capable of conveying desirable
properties to the biologically active polypeptidic
groups -- such as reduced immunogenicity, increased

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solubility, or reduced clearance rate from the body -- without significantly reducing the affinity of a given R_1 or R_2 group to its ligand. In the most preferred embodiments, the compound R_1 -X- R_2 (wherein $R_1=R_2$ and R_1 and R_2 are binding groups) has an affinity for its
5 ligand that exceeds the affinity that the non-derivitized binding group has to the ligand. For example, sub-stantially purified c105 30kDa TNF inhibitor PEG₃₄₀₀db has an inhibitor activity for TNF
10 that is greater than 20 times the inhibitor activity that c105 30kDa TNF inhibitor has for TNF.

The activating groups Y_1 and Y_2 that are part of the polymeric spacer X may be comprised of any of the activating groups as discussed above, including the
15 maleimide group, sulfhydryl group, thiol, triflate, tresylate, aziridine, oxirane, and 5-pyridyl. The preferred activating groups are maleimides.

The polymeric group $(Z)_n$ is preferably selected from the group consisting of polyethylene glycol, polypropylene glycol, polyoxyethylated glycerol, dextran, poly β -amino acids, colonic acids or other
20 carbohydrate polymers and polymers of biotin derivatives. In the preferred embodiments, the polymeric group is polyethylene glycol. Any non-peptidic polymeric group that would serve the functions
25 as described herein would also be included within the scope of this invention.

One of the advantages of the present invention is the ability to vary the distance between the groups R_1 and R_2 by varying the length of the polymeric group
30 linking the two binding groups. Although not limited by theory, it is proposed that the increase in biological activity seen for the multimeric compounds of this invention may be attributed to the multimeric
35 nature of the cell receptors and ligands in vivo. For this reason, the optimal distance between the units R_1 and R_2 (which would be generally directly proportional

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to the length of the polymeric unit $(Z)_n$) may be easily determined by one skilled in the art by varying the size of the spacer X.

5 In one embodiment of the present invention, the groups R_1 and R_2 are the same. However, in an alternate embodiment R_1 and R_2 are different species. Such compounds can be designed to create a heterodimer wherein both R_1 and R_2 act within the same general biological systems. For example, both IL-1 receptor
10 antagonist and TNF inhibitors are believed to disrupt the inflammation cascade. The difunctional complexes may also be designed where R_1 or R_2 is a "targeting" species that "directs" the complex to a specific location by its binding affinity to a certain
15 substrate, and the opposing binding group has a desired activity at the localized site.

An example of a heterodimer that has great potential for being a successful IL-2 inhibitor is one where R_1 is IL-2 α and R_2 is IL-2 β . Such a
20 heterodimer mimics the receptor complex that has the highest affinity for IL-2. See Example XVII. An additional heterodimer that can act as a complement inhibitor is the heterodimer where R_1 is the C3b binding domain from CR1 and R_2 is the C4b binding
25 domain from CR1. See Example XVIII. In an additional heterodimer R_1 is the exon 6 peptide of PDGF and R_2 is IL-1 α . See Example XIX.

In the preferred embodiment of the invention, the procedures for producing the bifunctional R_1 -X- R_2
30 complexes are essentially the same as those used for the site-selective reaction of polypeptides as described above. The synthesis of c105 30kDa TNF inhibitor PEG₃₄₀₀db is described below in Example 13. A bis-reactive polymeric group is reacted with a
35 cysteine-containing polypeptide, wherein the activating group on the bis-reactive polymeric group forms a thio-ether bond with the selected free cysteine residue. As described above, the cysteine may be a free cysteine

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naturally-occurring on the polypeptidic group, or a non-native cysteine that has been added or substituted into the natural sequence.

5 The preferred bis-reactive polymeric compound of the present invention is α -(2-maleimido) ω -maleimido poly(oxyethylene) or bis-maleimido PEG. The synthesis of bis-maleimido PEG is described in Example 12. According to the preferred method, the bis-maleimido compound is prepared from bis-hydroxyl PEG via the bis-
10 amino intermediate.

Several methods for the conversion of the terminal hydroxyls of PEG to the corresponding amino group have been reviewed by Harris et al., J. Polymer Sci. vol. 22, pg. 341 (1984); Harris, Rev. Macromol. Chem. vol. c25(3), pg. 325 (1985). This is
15 accomplished by generating a reactive intermediate via either sulfonation, halogenation, or oxidation of the hydroxyl followed by displacement of the activated termini by a nucleophile.

20 Other practical alternatives to the synthesis of the bis-maleimide PEG given in Example 12 also exist. The reactive intermediate in the conversion of the hydroxyl to the amine may be the halogenated derivative (e.g. the α -(bromoethyl)- ω -
25 bromopoly(oxyethylene) intermediate (Johannson, Biochim. et Biophys. vol. 222, pg. 381 (1970)) followed by direct substitution with ammonia, (Buckmann et al., Makromol. Chem. vol. 182, pg. 1379 (1981)) or the aldehyde intermediate (Harris, supra.) The bis-
30 maleimide PEG is not the only sulfhydryl-specific reagent that may be used. Glass and coworkers have developed another method for the attachment of PEG to sulfhydryls. Glass et al., J. Biopolymers vol. 18, pg. 383 (1979). However, the reaction is reversible with
35 thiols. Another method for attachment of PEG to cysteinyl sulhydryls is the bis-4-vinylpyridine PEG derivative.

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Harris (supra) also reviews the synthesis of a variety of electrophilic derivatives of PEG that can be used as reagents to modify proteins. The reagents include chlorocarbonates, isocyanate, epoxide, succinimidyl succinate, cyanuric chloride, mixed anhydride, carbodiimides and sulfonates. The latter group includes tresylate, tosylate, and mesylates. Some of the reagents react selectively with amines (e.g., cyanuric chloride and carbodiimides) while others react with both sulhydryls and amines (e.g., epoxide and tresylates). Some of these reagents have been used to modify proteins and may result in varying degrees in loss of activity.

The preferred preparation of R_1 -X- R_2 complexes where R_1 and R_2 are different requires a two step process where the bis-reactive polymeric group is reacted in series with R_1 and then R_2 . The preparation of such heterodimers may be accomplished by those of ordinary skill in the art without undue experimentation. In some cases the intermediate R_1 -X must first be isolated and purified prior to reaction with R_2 , and in other circumstances an intermediate purification may not be necessary.

The extracellular domains of both IL-2 α and IL-2 β may be cloned using PCR and cloned into a vector capable of directing expression in E. coli. The proteins may be refolded and purified from E. coli and their ability to inhibit IL-2 activity measured in bioassays. In vitro mutagenesis can be used to substitute native residues in the molecules with cysteine to allow for site directed attachment of PEG. Muteins of both IL-2 α and IL-2 β may then be identified that allow for efficient attachment of PEG which do not lose activity when PEGylated. A PEG-linked heterodimer may be formed by first PEGylating IL-2 α in the presence of an excess of bis-maleimido PEG. The singly PEGylated IL-2 α may be purified and

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IL-2r β added to react with the active maleimide group and form the heterodimer. This molecule may be purified and its activity assessed. This molecule should mimic the high affinity IL-2 receptor found on cell surfaces.

A dumbbell complex where R₁ is IL-2 and R₂ is IL-2r β should also be useful as a receptor antagonist of IL-2.

EXAMPLE I. SYNTHESIS OF POLYETHYLENE GLYCOLATING

AGENTS

Three reagents are described to indicate the diverse means that may be used to derivatize polypeptides. See, Appendix to Example 1, for structures of Intermediates and reagents described below. All references provided below are specifically incorporated herein by this reference.

A. SYNTHESIS OF REAGENT 1: mPEG_x-ESTER-MALEIMIDE

The succinate ester derivative of the mPEG_x (intermediate 1) was prepared as described by Wie et al. Int. Archs. Allergy App. Immun., vol. 64, pp. 84-99 (1981). The resulting product was weighed out and dissolved in a minimum of dry dioxane at 60°C. After the solution had cooled to ambient temperature, equimolar amounts of both tri-n-butylamine and isobutyl chloroformate were added. The reaction proceeded thirty minutes with stirring. During this time, a borate buffer, pH 8.8, was made by titrating a solution of 0.5 M boric acid with 1,6-hexanediamine. The solution containing the mixed anhydride was added dropwise to an aliquot of the borate buffer containing a 10-fold molar excess of 1,6-hexanediamine over the mixed anhydride. The reaction mixture was exhaustively dialyzed versus deionized water at 4°C and lyophilized. This polymer intermediate (intermediate 2) was reacted with a 2.5:1 molar excess of sulfosuccinimidyl 4-(N-

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maleimiodethyl) cyclohexane-1-carboxylate (sulfo-SMCC, Pierce Chemical Co., Rockford Ill.) in 50 mM sodium phosphate or HEPES buffer, pH 7.0, for two hours at room temperature. The resulting polymer was purified by size exclusion chromatography of the reaction mixture on Sephadex G-25 using 50 mM sodium phosphate (or HEPES) pH 7.0 for elution at 4°C. The maleimido-polymer (reagent 1) eluted at the void volume of the column and was detected by monitoring its absorbance at 260 nanometers. The reagent was used to alkylate polypeptides within one hour of its purification. Since the mPEG from this reaction can be removed by base hydrolysis, this reagent is useful for identifying the site of mPEG attachment to the protein.

15

B. SYNTHESIS OF REAGENT 2: mPEG_x-AMIDE
MALEIMIDE

The mPEG_x-tosylate (intermediate 3) was prepared as described by Pillai *et al.* J. Org. Chem. vol. 45, pp. 5364-5370 (1980). The amount of sulfonated intermediate was estimated spectrophotometrically as described by Nilson and Mosbach, in Methods of Enzymology, vol. 104, pp. 56-69, Academic Pres. Inc., N.Y., N.Y. (1984). This intermediate was converted to the phthalimide derivative (intermediate 4) and subsequently reduced with hydrazine hydrate to the mPEG_x-NH₂ intermediate (intermediate 5) by the procedure of Pillai *et al.*, *supra*. The amino group capacity in equivalents per gram of product was quantified by microtitration with hydrochloric acid. The mPEG_x-NH₂ was reacted with sulfo-SMCC in HEPES or phosphate buffer pH 7.2 at room temperature for two hours. The amount of the mPEG_x-amine to sulfo-SMCC was tested at molar ratios of 5:1 to 1:5.

35

To determine the optimal conditions the final reagent (reagent 2) was used in pegylation reactions and the quantity and quality of mPEG_x-IL-1ra (we will

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use this designation for the pegylated product of IL-1ra reacted with reagent 2 and mPEG_xIL-1ra for pegylated IL-1ra from a reaction with reagent 3 described below) obtained from these reactions was assessed by SDS-polyacrylamide gel electrophoresis (PAGE). The optimal result was seen with a 1:1 ratio of SMCC to mPEG_x-NH₂. Higher proportions of sulfo-SMCC generated multiple higher molecular weight derivatives of IL-1ra on SDS-PAGE and multiple peaks on analytical ion exchange chromatography and lower proportions resulted in a reduced yield of pegylated protein. Reagent 2 was purified by size exclusion chromatography using G25 sephadex resin.

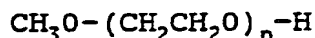
15 C. SYNTHESIS OF REAGENT 3: mPEG_x-MALEIMIDE
The mPEG_x-NH₂ (intermediate 5) can be modified further to yield a different maleimido-derivative (reagent 3). The latter was accomplished by reacting the mPEG_x-NH₂ with maleic anhydride via an adaptation of the procedure of Butler and Hartley, in Methods of Enzymology, vol. XXV pp. 191-199, Academic Press. Inc., N.Y., N.Y. (1972) and cyclizing this intermediate (intermediate 6) to the corresponding O-(2-maleimido ethyl)-O'-methylpolyethylene glycol using the method described by Wunsch et al., Biol. Chem. Hoppe-Seyler, vol. 366, pp. 53-61 (1985).

APPENDIX TO EXAMPLE I

SYNTHESIS OF REAGENT 1

30 Structures of starting material, intermediates and reagent from synthesis 1.
Starting material:
Generalized formula for monomethoxypolyethylene glycol (mPEG_x):

35



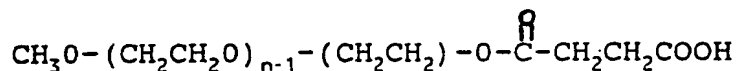
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where x denotes the average molecular weight of the polymer in kilodaltons and n is the average number of repeating oxyethylene groups.

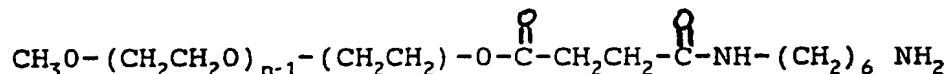
Intermediate 1:

5



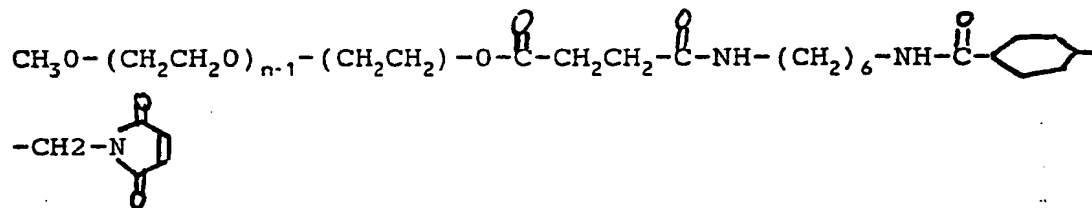
Intermediate 2:

10



Reagent 1:

15



SYNTHESIS OF REAGENT 2

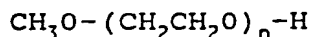
20

Structures of starting material, intermediates and reagent from synthesis 2.

Starting material:

Generalized formula for monomethoxypolyethylene glycol (mPEGx):

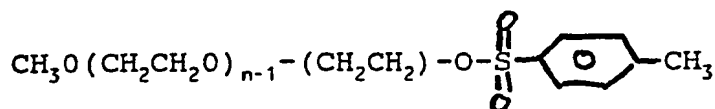
25



where x denotes the average molecular weight of the polymer in kilodaltons and n is the average number of repeating oxyethylene groups.

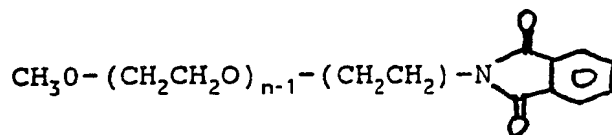
Intermediate 3:

30



Intermediate 4:

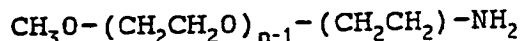
35



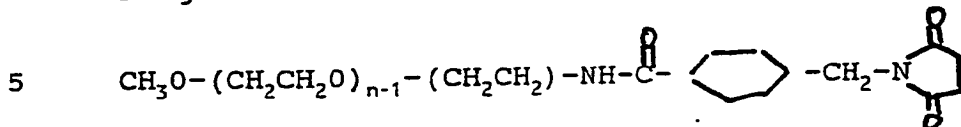
Intermediate 5 (mPEGx-NH₂):

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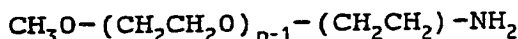
Reagent 2:



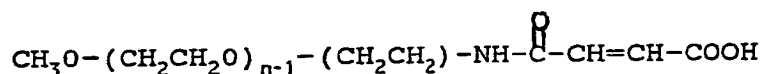
SYNTHESIS OF REAGENT 3

Structures of starting material, intermediates and reagent from synthesis 3.

10 Starting material:

Intermediate 5 (mPEG_x-NH₂):

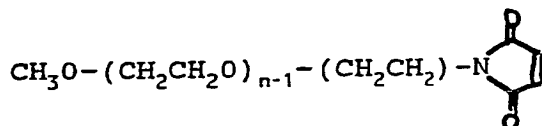
15 Intermediate 6:



Reagent 3:

20

O-(2-maleimidoethyl)-O'-methyl polyethylene glycol

25 EXAMPLE II. PREPARATION OF PEGYLATED NATIVE IL-1ra

Various parameters were tested in optimizing the pegylation reaction of native IL-1ra with successful pegylation assayed by visual inspection for a single tight band at 29 kilodaltons on Coomassie stained SDS-PAGE and a single sharp peak by analytical ion exchange chromatography. Unless otherwise stated, pegylation reactions were done at 1 mg/ml of native IL-1ra at room temperature in HEPES buffer pH 7.2 with a mPEG reagent to IL-1ra ratio of 2:1. The reagent used in these studies was mPEG-amido-maleimide (Reagent 2) and the product is referred to as mPEG_x*IL-1ra but the results are applicable to all three reagents.

30

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A. TIME

Pegylation reactions at room temperature were analyzed from 0.5 to 24 hours. Conversion of the IL-1ra to the pegylated form is complete (80%-90%) in two to four hours and the total amount of mPEG*IL-1ra does not increase or decrease after longer periods of incubation. The quality of the mPEG*IL-1ra assayed by SDS-PAGE decreases at longer times due to the appearance of additional bands and smears at higher molecular weights on the stained gel.

B. TEMPERATURE

Pegylation reactions were incubated at temperatures of 4°, 25°, 37°, and 50°C and then analyzed at time points of 0.5, 1, 2, 4 and 17 hours. The reactions at 25° and 37° generated a large amount (about 50%-80%) of pegylated protein within one to two hours but those at 4°C and 50°C resulted in a much lower yield (10%-20%) even at the later time points. The quality of the mPEG*IL-1ra does not seem to change significantly with temperature.

C. PROTEIN CONCENTRATION

Pegylation reactions have been done with protein concentrations (native IL-1ra) between 50 ug/ml and 10 mg/ml. All of the concentrations tested worked well and there was no difference in the quality of the mPEG*IL-1ra.

D. pH

Native IL-1ra was pegylated under the reaction conditions stated above between pH 5.5 and 7.5. The quality of the mPEG*IL-1ra is slightly better by SDS-PAGE and ion exchange at a lower pH (5.5) but the percent conversion is the same.

E. mPEG-AMIDO-MALEIMIDE TO NATIVE IL-1ra RATIO

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We tested ratios of between 0.5:1 to 20:1 of the mPEG-amido-maleimide to native IL-1ra. Ratios higher than about 2:1 result in efficient conversion to the pegylated form of IL-1ra (50%-90%). Ratios greater than 5:1, however, generate lower quality mPEG*IL-1ra by increasing the amount of extra high molecular weight bands on reduced SDS-PAGE and multiple peaks on ion exchange chromatography.

The optimal reaction conditions for both quantity of mPEG*IL-1ra obtained and quality of the material, within the parameters used, is a 2:1 mPEG-amido-maleimide/IL-1ra at 25°C for 2-4 hours using mPEG-amido-maleimide generated with a 1:1 ratio of Sulfo-SMCC to mPEG-amine. With these conditions 80-90% of the IL-1ra is converted to the pegylated form using reagent synthesized with either mPEG₅₀₀₀ or mPEG₈₅₀₀ as the starting material (Figure 3).

F. PREPARATION OF IL-1ra PEG DUMBBELLS

PEG dumbbell complexes containing IL-1ra are made according to the same procedures as other PEGylated IL-1ra species. A 2-4 molar excess of bis-maleimido PEG to IL-1ra in HEPES buffer at 7.0 is used. With IL-1ra, the species used may be the wild type molecule, which has a free and available cysteine residue, or a mutein prepared as described herein. The IL-1ra is at a concentration of 2-5 mg/ml. The reaction is incubated at ambient temperatures for 4 to 6 hours. The IL-1ra PEG dumbbell compounds are purified from the unPEGylated and singly PEGylated species by MonoS cation exchange at pH5.5 in 20-50mM MES buffer using a gradient from 0 to 1000 mM NaCl. Further purification may be achieved by size exclusion chromatography using a BioRad TSK 250 or Superdex 75 column, as described below.

EXAMPLE III. PURIFICATION OF PEGYLATED NATIVE IL-1ra

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Purification of mPEG_x*IL-1ra can be achieved by cation exchange or size exclusion chromatography. These procedures are applicable to pegylated IL-1ra derived from all three reagents described above.

5

A. CATION EXCHANGE CHROMATOGRAPHY

The mPEG_x*IL-1ra can be purified using a MonoS (Pharmacia) column with 20mM MES buffer at pH 5.5. The proteins were eluted from the column using a salt gradient from 0 to 500mM NaCl in the same buffer. For example, unmodified IL-1ra elutes at 220mM NaCl, while the purity is assessed by various techniques including analytical ion exchange chromatography and SDS-PAGE. mPEG₅₀₀₀ IL-1ra elutes at 160mM (Figure 4).

15

B. SIZE EXCLUSION CHROMATOGRAPHY

The mPEG₅₀₀₀*IL-1ra, which runs as about 52 kd, and mPEG₈₅₀₀*IL-1ra, which runs as about 68 kd (based on column calibration with known size standards), can easily be separated from unmodified IL-1ra (17 kd) by size exclusion chromatography on a Superdex 75 (Pharmacia) column with standard chromatographic techniques (Figure 5).

20

25 EXAMPLE IV: CHARACTERIZATION OF THE mPEG_x*IL-1ra

Purified mPEG_x*IL-1ra gave a single symmetrical peak upon rechromatography on MonoS and appeared pure by both SDS-PAGE and size exclusion chromatography (Figure 3 and 4). A comparison of the tryptic maps of IL-1ra and mPEG₅₀₀₀*IL-1ra showed one peak, corresponding to the peptide containing c116 and c122, absent from the conjugate map with the appearance of a new broad peak in this map. Subdigestion of this new peak with chymotrypsin and subsequent amino acid sequence analysis indicated that c116 had been pegylated under the conditions employed (Figure 6).

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EXAMPLE V. PREPARATION OF IL-1ra MUTEINS

Mutagenesis was performed on single stranded DNA from the IL-1ra gene cloned into the bacteriophage M13. BioRad's Mutagene kit was used which uses the
5 procedure described by Kunkel et al. Methods in Enzymology vol. 154, pp. 367-382 (1987). Briefly, single stranded DNA template was generated using an E. coli strain that contains the dut and ung mutations, resulting in template that contains uracil instead of
10 thymidine. Mutagenic oligonucleotides between 20 and 30 base pairs in length were annealed to the template and the second strand was resynthesized using DNA polymerase and DNA ligase. The reaction mixtures were used to transform a wild type E. coli strain in which
15 the uracil containing strand is degraded by the DNA repair mechanisms and the mutant strand is allowed to replicate. The mutant phage were screened and sequenced by standard techniques. The fragment containing the mutant gene was then subcloned into the
20 expression vector pT5T (Eisenberg et al. Nature vol. 343, pp. 341-346, (1989)) and transformed into the T7 expression system strain (E. coli B121DE3). Other E. coli expression systems may also be used.

Expression clones were grown in Luria Broth
25 supplemented with 15ug/ml tetracycline at 37°C. When the cultures reached an optical density of 0.8 at 600nm they were moved to 30° and IPTG was added to a final concentration of 1 mM to induce expression of the IL-1ra gene. Total accumulation of the IL-1ra protein was
30 maximal after 4-6 hours and did not change significantly for up to 12 hours post induction.

EXAMPLE VI: PURIFICATION OF THE IL-1ra MUTEINS

Cell cultures induced as described above were
35 harvested by centrifugation at 10000g for 10 min. The cells were resuspended in 30mM sodium acetate buffer pH 5.2 in 20-50 mls. Lysis was achieved by two passes

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through the French Pressure cell at 18000 psi. The cell lysate was centrifuged at 10000g for 10 minutes. The soluble portion was loaded onto a S-Sepharose column and washed with the same buffer containing 75mM NaCl. The IL-1ra mutein eluted with buffer containing 200mM NaCl. The single pass over the ion exchange resin resulted in a product of sufficient purity (>95%) for pegylation studies. Further purification can be achieved using other ion exchange resins such as Q-Sepharose or MonoQ. This procedure was used for several of the IL-1ra muteins with equal success. In some cases it was necessary to vary the pH and/or NaCl concentrations slightly to purify muteins which have a small change in protein charge due the change in amino acid sequence. With these slight variations that would be easily manipulated by one of ordinary skill in the art, this procedure is generally applicable to all of the muteins studied.

20 EXAMPLE VII: IL-1ra MUTEIN PEGYLATION

In addition to the native IL-1ra, muteins c84s116, c84c116, c0s116 and c9s116 were pegylated. Employing the same conditions used for the native IL-1ra, the pegylated forms of c84s116 and c84c116 were produced and purified. Since c84c116 contains two reactive cysteines, pegylation results in a higher molecular weight protein at about 40 kd on SDS-PAGE. This protein can be purified by cation exchange or size exclusion chromatography and runs at the expected molecular weight of about 68 kd on the latter when using PEG₅₀₀₀.

30 EXAMPLE VIII. EFFICACY OF THE mPEG*IL-1ra

The efficacy of the pegylated native IL-1ra molecules was tested by a standard competitive receptor binding assay using S³⁵-IL-1ra as the ligand. Mouse cells (EL4) containing the mouse type 1 IL-1 receptor

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or hamster cells (CHO) expressing from a cloned gene the human type 1 receptor were used at 1×10^6 cells per well and 1×10^5 cells per well, respectively, in 96 well microliter dishes. S^{35} -IL-1ra with a specific activity of 4000 Ci/mmol was added to a final concentration of 150pM. Cold ligand was added in serial dilutions from 28 mM to 13 pM and allowed to incubate for 4 hours at 4°C. The cells were then filtered through a Milliliter filter plate (Millipore, .5 micron pore size Durapore filter), washed to remove nonspecifically bound counts, the filter removed and counted on an Ambis Radioanalytical Imaging System. Equilibrium dissociation constants (kDs) were calculated and used to compare the pegylated and unmodified forms of IL-1ra. Unmodified wild type IL-1ra and c84s116 have equal kD's for the type 1 mouse receptor of 150-300 pM in our assay. The kD for the IL-1ra pegylated form is about 400-800 pM and for pegylated c84s116, 500-1000 pM which is 2.5 and 3.5 fold higher than that of the unmodified protein respectively. The kDs for all but one (c6s116) of the unpegylated muteins are within 65-150% of the native protein, within the standard error of the assay. See Table 1.

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TABLE 1
ANALYSIS OF PEGYLATED IL-1ra MOLECULES

		SIZE (kd)	RECEPTOR ASSAY
5	MUTEIN		
	WILD TYPE	17.5	100
	C84S116	17.5	98
10	C9S116	17.5	67
	C6S116	17.5	37
	C0S116	17.5	63
	C84C116	17.5	95
15	PEG*IL-1ra		
	SINGLE		
	PEG ₅₀₀₀ C116	50-60	34
	PEG ₅₀₀₀ C84s116	50-60	28
20	PEG ₈₅₀₀ C116	70-80	30
	PEG ₈₅₀₀ C84s116	70-80	30
	PEG ₈₅₀₀ C0s116	ND	22
	PEG ₈₅₀₀ C9s116	ND	12
	PEG ₁₂₀₀₀ C116	78	20
25	DOUBLE		
	PEG ₅₀₀₀ C84c116	70-80	11
	PEG ₈₅₀₀ C84C116	150-200	4
30	PEG ₁₂₀₀₀ C84c116	175	5
	DUMBBELLS		
	PEG ₃₅₀₀ C116	55-65	49
35	PEG ₃₅₀₀ C84	60	49
	PEG _{10,000} C116	175-200	49
	PEG _{10,000} C84	200	60
	PEG _{20,000} C84	>200	24
40	Data are presented as a percent of the activity exhibited by unmodified IL-1ra. Standard deviations are within 10%.		

45 EXAMPLE IX: PHARMACOKINETICS OF PEGYLATED NATIVE MUTEIN IL-1ra

50 The pharmacokinetic character of several pegylated native and mutein IL-1ra molecules was tested following intravenous injection of the molecules to rats. Native or pegylated IL-1ra was injected as an intravenous bolus dose (3 mg/kg). Serial blood samples

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were drawn from the tail vein and assayed for native or pegylated IL-1ra by enzyme-linked immunosorbent assay (ELISA). The resulting plasma IL-1ra concentration vs. time profiles (Figure 8) illustrate that pegylation has a pronounced influence on the disappearance of IL-1ra from the plasma after intravenous injection. The declines in plasma IL-1ra and pegylated derivatives of IL-1ra are best described by three exponential components. The data indicate that pegylation prolongs the half-lives of these exponential components up to six-fold in the rat (Table 2). The half-lives of these exponential components increase as the size of the PEG molecule increases (Table 2). Additionally, there is evidence that the prolongation of the half-lives may be pegylation site-specific. Standard compartmental analysis was used to interpret the data of Figure 8. The prolongation of half-lives may be explained based on accepted pharmacokinetic theory which states that the plasma half-life for a drug is inversely related to the plasma clearance for the drug and directly related to the apparent volume of distribution for the drug. Pharmacokinetic analysis of the disappearance of pegylated IL-1ras from the plasma indicate that the prolongation in half-life is inversely related to a decreased plasma clearance for the pegylated molecules, compared to native IL-1ra (Table 2). The decrease in plasma clearance is consistent with an anticipated size-related decrease in glomerular filtration of the pegylated molecules by the kidneys. Also, the prolongation of the half-lives by pegylation is directly related to an increase of the distribution (Vd steady-state, Table 2) of the pegylated molecule. The increase in distribution volume indicates greater penetration of the pegylated molecules into the extravascular pool. Through this mechanism pegylation improves therapy with IL-1ra by increasing the extent

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to which the active molecules move from the systemic circulation into the extravascular compartment, a compartment in which IL-1 receptors are expected to be located. Because of the similarity between rats and humans in both clearance and distribution mechanisms for IL-1ra, it is apparent that pegylation will similarly improve the pharmacokinetic properties of IL-1ra in humans.

10 1. Additional intravenous pharmacokinetics for pegylated IL-1ra

 The intravenous pharmacokinetics for eight additional pegylated IL-1ra muteins have been characterized using methods previously described. A plot containing intravenous plasma IL-1ra concentration vs. time curves for each of the molecules is attached (Figure 10). Review of all of the intravenous pharmacokinetic data (Table 3) indicates that as the size of the PEG (single or double) is increased, the plasma clearance decreases and hence the intravenous mean residence time and plasma IL-1ra disappearance half-lives increase. The site of pegylation is important in determining the extent to which the pegylation decreases the plasma clearance and prolongs the means residence time. The addition of two PEGs to IL-1ra prolongs the intravenous mean residence time fourteen-fold compared to wild type IL-1ra.

2. Subcutaneous pharmacokinetics for pegylated IL-1ra

30 Absorption pharmacokinetics of pegylated IL-1ra muteins have been characterized following subcutaneous injection of the molecules to rats. Serial blood samples were drawn from the tail vein and assayed for native or pegylated IL-1ra by enzyme-linked immunosorbent assay (ELISA). The resulting subcutaneous plasma IL-1ra concentration vs. time curves are plotted in Figure 11. The subcutaneous

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pharmacokinetic data (Table 3) reveal variable systemic availability for the pegylated muteins, related to the site and size of the PEG, and related to subcutaneous injection in non-optimized formulations. Table 3 also reveals a remarkable positive influence of pegylation on the mean residence time for subcutaneously injected IL-1ra. As the size of the PEG is increased, the mean residence time is generally increased. This increase is probably the result of molecule-size-related slower absorption through the lymphatic circulation (longer mean absorption times) as well as to delayed clearance after the pegylated molecule reaches the systemic circulation (plasma). This prolongation is profound and will improve the pharmacokinetic character of subcutaneous IL-1ra in humans.

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EXAMPLE X: PREPARATION OF 30kDa TNF INHIBITOR MUTEINS

Cysteine has been substituted for the native residue at both the amino terminus and carboxyl terminus of the protein as well as all three glycosylation sites (residues 1, 14, 105, 111 and 161 as seen in Figure 2). Mutagenesis was performed on single stranded DNA from the 30kDa TNF inhibitor gene cloned into the bacteriophage M13. This gene is described in detail in U.S. Patent Application Serial No. 07/555,274 filed July 19, 1990. Mutagenesis was done as described by Kunkel *et al.* (1987) (see Example V). The mutagenized gene was isolated and subcloned into the expression vector pT5T (Eisenberg *et al.*, *Nature* vol. 343, pg. 341 (1989)) and transformed into the T7 expression system strain *E. coli* BL21DE3. The 30kDa TNF inhibitor muteins were purified and refolded as described for native 30kDa TNF-inhibitor. See, the U.S. Patent Application Serial No. 07/555,274 filed July 19, 1990. Refolding includes the addition of cysteine to the solution containing the purified protein. The cysteine aids in the refolding and "bonds to" the free cysteine in the mutein.

EXAMPLE XI: PEGYLATION OF 30kDa TNF INHIBITOR MUTEINS

The c105 30kDa TNF Inhibitor mutein was exposed to a 6-fold molar excess of DTT in 50mM HEPES Ph 7.0 for 30 minutes at ambient temperature in order to remove an extra cysteine attached during the refolding process. The protein was then dialyzed against degassed 50mM HEPES pH 7.0 for 2 hours to remove the DTT. The c105 30kDa TNF inhibitor was then reacted with a 5 fold molar excess of pegylating reagent 1 (See Example 1A) for 2 hours at ambient temperature in 50mM HEPES pH 7.0. Approximately 60% of the mutein was converted to the pegylated form.

The c105 pegylation reaction mixture was loaded onto a superdex-75 FPLC column (Pharmacia) run at 0.25

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ml/min in 50mM Tris pH 7.0, 100mM NaCl. Fractions containing c105-PEG 30kDa TNF-inhibitor were pooled and loaded on a TSK-2000SW HPLC column (Bio-Rad) run at 0.2ml/min in the same buffer. The fractions containing essentially pure c105-PEG 30kDa TNF-inhibitor, as determined by silver stained SDS-PAGE, were pooled and the protein concentration determined by Bio-Rad protein assay. See Figure 9.

The activity was determined using the murine L929 cell TNF cytotoxicity assay as described in U.S. Patent Application Serial No. 07/555,274 filed July 19, 1990.

EXAMPLE XII: THE PREPARATION OF BIS-MALEIMIDO PEG

The synthesis of the α -(2-aminoethyl) ω -aminopoly(oxyethylene) derivative of the PEG (hereinafter bisamino PEG) consisted of three steps: 1) sulfonation of the hydroxyl group using tresyl chloride as described by Nilson and Mosback (Nilson *et al.*, Methods in Enzymology vol. 104, pg. 56, Academic Press, Inc., N.Y., N.Y. (1984)), 2) substitution of the tresylated intermediate by phthalimide (Pillai *et al.*, J. Org. Chem. vol. 45, pg. 5364 (1980)), and 3) reduction of the phthalimide intermediate to amine by hydrazine hydrate (Pillai, *supra.*). Structures of the starting material, intermediates, and products are shown in Appendix 1 to this Example. Optimum conditions permitted a conversion of approximately 80% of the hydroxyl to amine as determined by 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay. The bisamino PEG can be purified from the reaction mixture by ion-exchange chromatography. This is a key step for removing reactive byproducts which can interfere with dimer formation.

The bisamino PEG was acylated using maleic anhydride (Butler *et al.*, Methods in Enzymology vol. 25, pg. 191, Academic Press, Inc., N.Y., N.Y. (1972))

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and the resulting intermediate was cyclized to produce α -(2-maleimidoethyl)- ω -maleimidopoly(oxyethylene) (Winsch *et al.*, Biol. Chem. Hoppe-Seyler vol. 336, pg. 53 (1985)). This derivative reacts with sulfhydryls via a Michael addition to form a stable thioether.

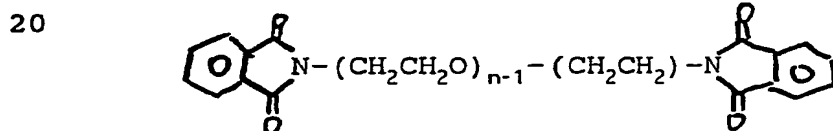
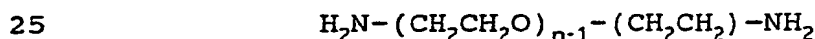
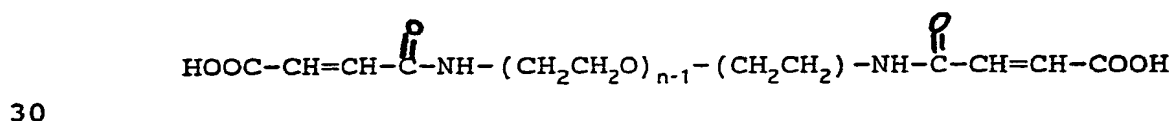
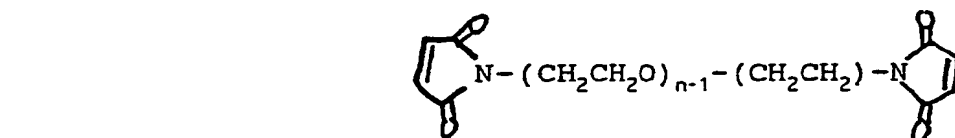
APPENDIX TO EXAMPLE XIIStarting Material

Generalized formula for polyethylene glycol PEG_x



where x denotes the average molecular weight of the polymer in kilodaltons and n is the average number of repeating oxyethylene groups.

15 Intermediate 1

Intermediate 2Intermediate 3Intermediate 4O-(2-maleimidoethyl)-O'-methyl-polyethylene glycol

EXAMPLE XIII: IN VIVO RESULTS FOR c105 30kDa TNF INHIBITOR PEG COMPLEXES

The inhibitory effects of four species of

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pegylated c105 30kDa TNF inhibitor species were tested in vivo on two different TNF-stimulated physiological actions. One endpoint was the appearance of IL-6 in the plasma of mice that were injected intravenously with human recombinant TNF. The second endpoint was an increase in the migration of neutrophils into the peritoneal cavity after the intraperitoneal administration of human recombinant TNF.

10 **Experiment One.** The intravenous administration of c105 30kDa TNF inhibitor (PEG_{2,000}, PEG_{3,500}, PEG_{10,000}) simultaneously with human recombinant TNF inhibits the induction of IL-6 in the plasma of mice.

15 BALB/c female mice weighing 20 to 23 g were used to measure the induction of plasma IL-6 levels by human recombinant TNF. In a preliminary experiment, the time course was plotted for the appearance IL-6 in the plasma after the intravenous administration via the tail vein of two doses of human recombinant TNF (Figure 20 12). Peak IL-6 levels occurred at two hours after stimulation with either 10 or 20 ug of human recombinant TNF per mouse. The lower dose was used in subsequent experiments.

25 The potency of c105 30kDa TNF inhibitor PEG₂₀₀₀ dumbbell with that of the unpegylated c105 30kDa TNF inhibitor was compared. Human recombinant TNF was injected intravenously at a dose of 10 ug per mouse either alone or simultaneously with the TNF inhibitors. Four different reactions of inhibitors to TNF were 30 tested (Figure 13). The ratios were calculated based on protein content. Three mice were tested at each dose. Blood was collected at two hours after the intravenous injections. IL-6 levels were measured by ELISA.

35 Both the c105 30kDa TNF inhibitor and c105 30kDa TNF inhibitor PEG₂₀₀₀ dumbbell caused nearly complete inhibition of IL-6 levels when administered at

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10:1 and 5:1 ratios of inhibitor to TNF. At ratios of 1:1, the c105 30kDa TNF inhibitor PEG₂₀₀₀ dumbbell caused 95% reduction of IL-6 levels stimulated by TNF alone, whereas the unpegylated c105 30kDa TNF inhibitor reduced IL-6 by only about 70%. The results of this experiment indicate that in the ratios tested, both the c105 30kDa TNF inhibitor and c105 30kDa TNF inhibitor PEG₂₀₀₀ dumbbell were good inhibitors of this TNF-stimulated physiological parameter. At a ratio of 1:1, the c105 30kDa TNF inhibitor PEG₂₀₀₀ dumbbell caused a greater percentage inhibition than the unpegylated inhibitor.

Two other species of pegylated c105 30kDa TNF inhibitor were tested. The inhibitory effects of c105 30kDa TNF inhibitor PEG_{3,500} dumbbell and c105 30kDa TNF inhibitor PEG_{10,000} dumbbell were tested on plasma IL-6 induction. The inhibitors were administered by intravenous injection simultaneously with human recombinant TNF at ratios of 1:1 (c105 30kDa TNF inhibitor dumbbell: TNF) (Figure 14). Three mice were tested in each of the two inhibitor-treated groups. Ten mice were injected with TNF alone. When administered in ratios of 1:1, no detectable IL-6 was measured in plasma of mice injected with either c105 30kDa TNF inhibitor PEG_{3,500} dumbbell or c105 30kDa TNF inhibitor PEG_{10,000} dumbbell, whereas a significant IL-6 response was elicited in the mice injected with human recombinant TNF alone.

The results of the two experiments show that c105 30kDa TNF inhibitor PEG_{2,000}, PEG_{3,500}, and PEG_{10,000} dumbbells are good inhibitors of the induction of plasma IL-6 by human recombinant TNF when administered in a low ratio (1:1) relative to the stimulus.

Experiment Two. The subcutaneous administration of c105 30kDa TNF inhibitor (PEG_{3,500}, PEG_{10,000} and PEG_{20,000}) simultaneously with the

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intraperitoneal injection of human recombinant TNF inhibits the migration of neutrophils into the peritoneal cavity.

5 BALB/c female mice weighing 20 to 23 g were used to measure the migration of neutrophils into the peritoneal cavity after stimulation with human recombinant TNF. The technique used is that of Kim McIntyre et al. (J. Exp. Med. vol. 173, pg. 931 (1991)) and is described in brief herein. Mice are injected
10 with TNF in a volume of 0.1 ml directly into the peritoneal cavity. Four hours later the mice are killed and an immediate post mortem lavage of the peritoneal cavity is performed. Four ml of Hank's Balanced Salt Solution (HBS) (calcium and magnesium
15 free) is injected into the peritoneal cavity. The abdomen is gently massaged. The peritoneal fluid is recovered by aspiration with needle and syringe. The total number of peritoneal cells is counted on a Coulter counter. An aliquot of the cellular suspension
20 is dried on a slide and stained with Diff-Kwik stain. A differential count of the cells is made by direct microscopic examination. One hundred cells are examined and classified as either neutrophils, lymphocytes, or macrophages.

25 In a preliminary experiment, the compared cellular make-up of the lavage fluid after intraperitoneal administration of either pyrogen-free saline or 7.5 ng human recombinant TNF was compared. TNF caused an increase in the percentage of neutrophils
30 and in the absolute number of neutrophils present in the peritoneal lavage fluid. In saline-treated mice, 9.4×10^4 neutrophils were recovered in the lavage fluid and made up only 2.3% of the total peritoneal cells. In TNF (7.5 ng)-treated mice, the total number
35 of neutrophils was increased to 12.9×10^5 and the percentage of neutrophils was increased to 19.7%.

The potency of unpegylated c105 30kDa TNF

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inhibitor with three pegylated species of c105 30kDa TNF inhibitor (PEG_{3,500}, PEG_{10,000} and PEG_{20,000} dumbbells) was also compared. Keeping the TNF stimulus constant at 7.5 ng per mouse, the inhibitors were tested at ratios of 100:1, 10:1, and 1:1 (c105 30kDa TNF inhibitor species: TNF). The ratios were calculated based on protein content. The mice were injected subcutaneously with the c105 30kDa TNF inhibitor simultaneous to the intraperitoneal administration of TNF. Six mice were tested in each dose group. Four hours later the peritoneal lavage fluid was collected and analyzed. Values shown in Figure 15 are the percentage neutrophils in the peritoneal lavage fluid. The lowest ratio at which the unpegylated c105 30kDa TNF inhibitor and c105 30kDa TNF inhibitor PEG_{3,500} dumbbell significantly inhibited neutrophil migration is 100:1. The c105 30kDa TNF inhibitor PEG_{10,000} and PEG_{20,000} dumbbells significantly inhibited neutrophil migration at a ratio of 10:1.

The results of this experiment show that c105 30kDa TNF inhibitor PEG_{3,500}, PEG_{10,000} and PEG_{20,000} dumbbells are good inhibitors of the TNF-stimulated neutrophilic migration into the peritoneal cavity. The c105 30kDa TNF inhibitors PEG_{10,000} and PEG_{20,000} dumbbells were more potent than the unpegylated c105 30kDa TNF inhibitor and the c105 30kDa TNF inhibitor PEG_{3,500}.

EXAMPLE XIV: PREPARATION AND BIOACTIVITY OF c105 30kDa TNF INHIBITOR PEG DB

Synthesis

Recombinant c105 30kDa TNF inhibitor 2-3 mg/ml is treated with a 4-fold molar excess of DTT for 2 hrs at ambient temperature. The TNF inhibitor is then dialyzed against de-gassed 50mM HEPES, pH 7.0, for 3 hrs at 4°C. To create the PEG-linked dumbbell, the TNF inhibitor is reacted with different molar ratios of the bis-maleimido PEG in 50 mM HEPES pH 7.0. TNF inhibitor

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is reacted with an equimolar ratio of bis-maleimido PEG. The reactions are incubated for 3-12 hrs at ambient temperature. After incubation, the PEG-linked TNF inhibitor dumbbell is purified from un-PEGylated and singly-PEGylated TNF inhibitor using MONO-S FPLC in 50 mM HOAc, pH 4.0, using a 260 mM, 310 mM and 350 mM NaCl step-gradient. The PEG-linked TNF inhibitor dumbbell elutes at the 310 mM NaCl step. Any remaining unPEGylated TNF inhibitor is removed by chromatography on Superdex75.

STEPWISE REAGENT ADDITION:

After DTT treatment and dialysis into 50 mM HEPES pH 7.0, an equimolar amount of bis-maleimido PEG is added, after 1.5 hrs incubation another equimolar amount of bis-maleimide PEG is added. This is incubated for 1.5 hours. This leads to an optimized level of PEG-linked dumbbell formation. Then a 2-fold excess of PEG reagent is added, giving a final PEG-TNF inhibitor ratio of 4:1. This is incubated for 2 hrs and the mixture is dialyzed into 50 mM acetate pH 4.0 for Mono-S chromatography. This yields a mixture which is primarily PEG-linked dimer and singly PEGylated TNF inhibitor. This allow for more efficient purification of PEG-linked dumbbell as there is a greater separation between singly PEGylated TNF inhibitor and dumbbell than dumbbell and unPEGylated TNF inhibitor.

This procedure optimized dumbbell formation, and allowed for more efficient purification.

STEP REACTION:

After DTT treatment and dialysis into 50 mM HEPES pH 7.0 an 8-fold molar excess of bis-maleimido PEG is added. This is incubated for 2 hrs at ambient temperature. This converts essentially all the TNF inhibitor to singly-PEGylated form. The singly-PEGylated TNF inhibitor is separated from PEG reagent

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and any remaining unreacted TNF inhibitor using MONO-S HPLC in 50 mM acetate pH 4.0 with a NaCl gradient. The singly-PEGylated material is diafiltered into 50 mM HEPES, pH 7.0, and concentrated to 2-4 mg/ml. DTT treated TNF inhibitor is then added to allow formation of PEG-linked dumbbell. After 2 hrs, the PEG-linked dumbbell is purified using Mono-S HPLC. This method may be used to form a PEG-linked heterodumbbell by adding a second, distinct protein compound.

This procedure optimizes dumbbell formation and can be used for the formation of heterodumbbell compounds. However, this procedure is somewhat labor and time intensive.

Bioactivity of PEG-linked TNF inhibitor Dumbbells

The ability of c105 30kDa TNF inhibitor dumbbells to inhibit the cytotoxicity of TNF α in the murine L929 cell cytotoxicity assay was measured. This has allowed for the determination of an ED₅₀ for these molecules.

They are as follows:

Wild Type rTNF inhibitor	220 ng/ml
BMH-linked dumbbells	220 ng/ml
1900 MW PEG-dumbbells	4.1 ng/ml
3500 MW PEG-dumbbells	4.8 ng/ml
10,000 MW PEG-dumbbells	4.6 ng/ml
20,000 MW PEG-dumbbells	4.2 ng/ml

The TNF inhibitor dumbbells also have greatly increased activity in inhibiting the cytotoxicity of TNF β in the L929 bio-assay. The ED₅₀ values against TNF β are as follows:

Wild Type rTNF inhibitor	70 μ g/ml
3400 MW PEG-dumbbells	80 ng/ml
20,000 MW PEG-dumbbells	22 ng/ml

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EXAMPLE XV: PHARMACOKINETICS OF PEGYLATED 30kDa TNF INHIBITOR1. Intravenous pharmacokinetics for pegylated 30kDa TNF inhibitor

The pharmacokinetic character of several pegylated 30kDa TNF inhibitor molecules was determined following intravenous administration of the molecules to rats. Native or pegylated TNF inhibitor was injected as an intravenous bolus dose. Serial blood samples were drawn from the tail vein and assayed for non-pegylated or pegylated TNF inhibitor by enzyme-linked immunosorbent assay (ELISA). The resulting intravenous plasma TNF inhibitor concentration vs. time profiles (Figure 16) illustrate that pegylation has a pronounced influence on the disappearance of TNF inhibitor from the plasma after intravenous injection. Statistical moment theory (area under the curve [AUC] and area under the first moment curve [AUMC]) was used to interpret the data of Figure 16. The data indicate that pegylation prolongs the intravenous mean residence time of TNF inhibitor up to fifty-fold in the rat (Table 4). The intravenous mean residence time increases as the size of the attached PEG molecule increases (Table 4). Although not limited by theory, the prolongation of mean residence times may be explained based on conventional pharmacokinetic theory which states that the intravenous mean residence time for a drug is inversely related to the plasma clearance for the drug and directly related to the apparent volume of distribution for the drug. Pharmacokinetic analysis of the disappearance of pegylated TNF inhibitor's from the plasma indicates that the prolongation of half-lives is inversely related to a decreased plasma clearance for the pegylated molecules, compared to non-pegylated TNF inhibitor (Table 4). The decrease in plasma clearance is consistent with an anticipated size-related decrease in glomerular

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filtration of the pegylated molecules by the kidneys. Because of the probable qualitative similarity between rats and humans in plasma clearance mechanisms for TNF inhibitor, it is apparent that pegylation will
5 similarly improve the pharmacokinetic properties of TNF inhibitor in humans.

2. Subcutaneous pharmacokinetics for pegylated 30kDa TNF inhibitor

10 Absorption pharmacokinetics of pegylated TNF inhibitor have been characterized following subcutaneous injection of the molecules to rats. Serial blood samples were drawn from the tail vein and assayed for non-pegylated or pegylated TNF inhibitor
15 concentration vs. time curves and are plotted in Figure 17. The subcutaneous pharmacokinetic data (Table 4) reveal variable systemic availability for the pegylated molecules, related to the size of the PEG, and related to subcutaneous injection in non-optimized
20 formulations. Table 4 also reveals a positive influence of pegylation on the mean residence time for subcutaneously injected TNF inhibitor. As the size of the PEG is increased, the mean residence time is generally increased. While not limited by theory, this
25 increase is likely the result of size-related slower absorption through the lymphatic circulation (longer mean absorption times) as well as delayed clearance once the pegylated molecule reaches the plasma. This prolongation is profound and will improve the
30 pharmacokinetic character of subcutaneous TNF inhibitor in humans.

EXAMPLE XVI. SOLUBILITY OF PEGYLATED PROTEINS

IL-1ra

35 Results of a solubility study are shown in Figure 18. Solubility curves are shown for three different preparations of IL-1ra, and c84 IL-1ra

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PEG₈₅₀₀. The experiments were preformed at 37°C in a microliter plate with all proteins at 160 mg/ml. The plate was sealed with a cover and then read in a plate reader at 405 nm at various time points. An increase in absorbance is an indication of protein precipitation. There is clearly a decrease in the amount of protein falling out of solution for the PEGylated sample relative to native IL-1ra.

30kDa TNF inhibitor

Native 30kDa TNF inhibitor cannot be concentrated to more than 5 mg/ml. Following PEGylation, the solubility was increased at least 5 fold.

EXAMPLE XVII: PREPARATION OF IL-2 INHIBITOR HETERODUMBELL

A PEG-linked heterodumbbell may be formed by first pegylating IL-2r α in the presence of an excess of bis-maleimido PEG. The singly pegylated IL-2r α may be purified and IL-2r β added to react with the remaining reactive maleimide group to form the heterodimer.

Potential sites for PEGylation of IL-2r α include both the amino and carboxyl terminal residues, the two N-linked glycosylation sites, as well as the native free cysteine residue in the molecule. Cysteine residue 192 in the soluble extracellular domain of IL-2r α has been identified as being uninvolved in disulfide bonding. (Miedel et al. BBRC, vol. 154, pg. 372 (1988)). This cysteine residue lies in an epitope of an anti-IL-2r α monoclonal antibody that does not affect IL-2 binding to IL-2r α (Lorenzo et al. J. Immunology, vol. 147, pg. 2970 (1991)). This indicates this residue is a likely candidate for PEGylation without affecting the activity of IL-2r α .

For IL-2r β , the potential sites include both the amino and carboxyl termini, the 4 N-linked glycosylation sites and a region (a.a. #108-118) that

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is similar to a region of biological significance in the murine erythropoietin receptor (Yoshimura, Longmore and Lodish, Nature, vol. 348, pg. 647 (1990)). Point mutational analysis of other residues in the receptors may also allow for identification of other sites of PEGylation that yield optimal properties in the heterodumbbell molecule.

10 EXAMPLE XVIII: PREPARATION OF HETERODUMBBELLS WHICH INHIBIT THE CLASSICAL PATHWAY OF COMPLEMENT SYSTEM

Many proteins which regulate the complement system have been identified and cloned. Some of them are membrane proteins. One of the membrane proteins is called CR1 (complement receptor 1). The soluble form of CR1 has been examined in in vivo models of diseases. The complement inhibitor inhibits post-ischemic myocardial inflammation and necrosis (Weisman et al. Science, vol. 149, pg. 145-151, 1990), reversed passive arthus reaction (Yet et al. J. Immunology, vol. 146, pg. 250-256 (1991)), and allograft rejection (Pruitt et al. J. Surgical Research, vol. 50, pp. 350-355 (1991)).

The soluble CR1 binds to C3b and C4b. It consists of 30 short consensus repeat sequences (SCR). Most of SCR contain one possible glycosylation site and four cysteines. All of the cysteines are likely be to involved in disulfide bonding. SCRs 1-4 are found to be involved in C4b binding. Two separate portions of CR1, SCRs 8-11 and SCRs 15-18, are involved in C3b binding (Klickstein et al. J. Exp. Med., vol. 168, pp. 1699-1717 (1988); Kalli et al. J. Exp. Med. vol. 174, pp. 1451-1460 (1991)). According to this invention, it is possible to produce a heterodumbbell which contains the C4b binding domain and the C3b binding domain of CR1.

The SCRs which contain C4b binding and C3b binding domains of CR1 may be cloned using PCR. These SCRs will be SCRs 1 through 5 (C4b binding) and SCRs 8

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through 12 (C3b binding). The genes encoding these SCRs may be cloned in E. coli expression vector. The E. coli expressed-proteins may be refolded and purified. The success of refolding can be analyzed by the capacity to bind polyC3b or polyC4b. In vitro mutagenesis of these genes may be carried out to substitute native amino acid residues to cysteine. These cysteines may then be used to link the PEG molecule. Possible sites for PEGylation will be the glycosylation site or carboxyl terminal residue of SCR 5 and SCR 12. The C4b binding and C3b binding domains which contain an extra cysteine to the carboxyl terminal residue could be constructed and used for linking PEG molecule. The PEG linked heterodumbbell may be produced by the two step process of Example XIV. Purification may be carried out by ion-exchange chromatography.

EXAMPLE XIX: SYNTHESIS OF AN IL-1ra BIS(MALEIMIDE)-PLATELET DERIVED GROWTH FACTOR PEPTIDE PEG HETERODUMBELL

The platelet derived growth factor (PDGF) peptide YGRPRESGKKRKRKRLKPT is described in Khachigian, L. et al. J. Biol. Chem., vol. 267, pg. 1660-1666 (1991). A terminal C was added to permit coupling to the maleimide.

The heterodumbbell was synthesized in two steps. In the first step, 1.6 nanomoles of IL-1ra suspended in 3 μ l of 0.05 M Hepes buffer, pH 7.5, was mixed with 6.4 nanomoles of bis-maleimido PEG₁₉₀₀ dissolved in 11 μ l of the same buffer. This reaction was carried out for 30 min at 20°C. In the second step, 32 nanomoles of the PDGF peptide dissolved in 4 μ l of 0.2 M sodium phosphate buffer, pH 7.0, was added to the products of the first reaction. The reaction was allowed to proceed for 1 hr at 20°C. The reaction was then terminated by the addition of an equal volume of SDS-PAGE sample buffer containing 30 μ moles of 2-

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mercaptoethanol.

Samples of the products of the first step of the reaction and the products of the complete two-step reaction, as well as appropriate molecular weight markers, were separated by SDS-PAGE on a 15% polyacrylamide gel which was then stained with Coomassie Blue. The two-step reaction gave an additional band consistent with the predicted size of the heterodumbbell. Approximately 33% of the starting IL-1ra was converted to heterodumbbell by the two-step reaction.

The products of the first step of the reaction can be isolated by cation exchange chromatography on the resin S-Sepharose. The heterodimer may be isolated by cation exchange chromatography due to the abundance of basic amino acids in the peptide.

It is to be understood that the application of the teachings of the present invention to a specific expression system or pegylation reagent will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Thus, it will be apparent to those of ordinary skill in the art that various modifications and variations can be made in the process and products of the present invention. It is intended that the present invention covers these modifications and variations provided they come within the scope of the appended claims and their equivalents.

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TABLE 4

	type of pegylation = none	none	single	single	dumbbell	dumbbell	dumbbell
	PEG location = [wild	C105	C105	C105	C105	C105	C105
	PEG size = type]	0	8500	20000	3500	10000	20000
intravenous pharmacokinetics							
number of animals =		2	2	2	2	2	2
Vd steady-state, mL/kg =		230	240	140	340	93	130
plasma clearance, mL/min/kg =		11.0	1.7	0.17	0.82	0.16	0.11
plasma mean residence time (i.v.), hr =		0.37	2.3	14	6.8	10	19
subcutaneous pharmacokinetics							
number of animals =	2		2	1	1	2	2
systemic availability, % =	99*		25	65	29	39	34
plasma mean residence time (s.c.), hr =	3.5		7.0	20	12	17	30
mean absorption time, hr =	3.1*		4.7	6.0	5.2	7.0	11

* referenced to C105 intravenous pharmacokinetics

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CLAIMS

1. A substantially purified compound of the formula R_1-X-R_2 wherein:
5 R_1 and R_2 are polypeptidic groups; and
 X is a non-peptidic polymeric spacer.
2. The substantially purified compound of claim 1 wherein R_1 and R_2 are the same group.
- 10 3. The substantially purified compound of claim 1 wherein R_1 and R_2 are different groups.
4. The substantially purified compound of claim 2
15 wherein R_1 and R_2 are selected from the group consisting of:
IL-1 receptor antagonist, 30kDa TNF inhibitor, 40kDa TNF inhibitor, IL-2 receptor, CR1, PDGF receptor, IL-2, MCSF receptor, EGF receptor, IL-5 receptor, IL-3
20 receptor, GMCSF receptor, T-cell receptor, HLA-I, HLA-II, NGF receptor, IgG (V_H , V_L), CF40, CD27, IL-6 receptor, Integrins CR3, VLA_4 , ICAM, and VCAM, CR2, GMP140 Lec domain, Laminin binding protein, Laminin fragments, Mannose binding protein, exon 6 peptide of
25 PDGF, and proteases.
5. The substantially purified compound of claim 4 wherein R_1 and R_2 are selected from the group consisting of: interleukin-1 receptor antagonist,
30 30kDa tumor necrosis factor inhibitor, interleukin-2 receptor, and CR1.
6. The substantially purified compound of claim 5 wherein R_1 and R_2 are interleukin-1 receptor
35 antagonist.
7. The substantially purified compound of claim 6

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wherein said interleukin-1 receptor antagonist is modified to contain at least one non-native cysteine residue.

5 8. The substantially purified compound of claim 7 wherein said non-native cysteine residue are found at amino acid residue sites selected from the group consisting of 0, 84, 6, 8, 9 and 141.

10 9. The substantially purified compound of claim 5 wherein R_1 and R_2 are 30kDA tumor necrosis factor inhibitor.

15 10. The substantially purified compound of claim 9 wherein said 30kDA tumor necrosis factor inhibitor is modified to contain at least one non-native cysteine residue.

20 11. The substantially purified compound of claim 10 wherein said non-native cysteine residue are found at amino acid residue sites selected from the group consisting of 1, 14, 105, 111 and 165.

25 12. The substantially purified compound of claim 1 where R_1 and R_2 are interleukin-2 receptors.

30 13. The substantially purified compound of claim 12 wherein said interleukin-2 receptor is modified to contain at least one non-native cysteine residue.

35 14. The substantially purified compound of claim 12 wherein R_1 is IL-2 α and R_2 is IL-2 β .

35 15. The substantially purified compound of claim 1 wherein R_1 and R_2 are CR1.

16. The substantially purified compound of claim 15

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wherein said CR1 is modified to contain at least one non-native cysteine residue.

17. The substantially purified compound of claim 3
5 wherein R_1 and R_2 are selected from the group consisting of:

IL-1 receptor antagonist, 30kDa TNF inhibitor, 40kDa TNF inhibitor, IL-2 receptor, CR1, PDGF receptor, IL-2, MCSF receptor, EGF receptor, IL-5 receptor, IL-3
10 receptor, GMCSF receptor, T-cell receptor, HLA-I, HLA-II, NGF receptor, IgG (V_H , V_L), CF40, CD27, IL-6 receptor, Integrins CR3, VLA_4 , ICAM, and VCAM, CR2, GMP140 Lec domain, Laminin binding protein, Laminin fragments, Mannose binding protein, exon 6 peptide of
15 PDGF, and proteases.

18. The substantially purified compound of claim 17
wherein R_1 and R_2 are selected from the group consisting of: interleukin-1 receptor antagonist;
20 30kDa tumor necrosis factor inhibitor; interleukin-2 receptor, and CR1.

19. The substantially purified compound of claim 1
wherein X is selected from the group consisting of:
25 polyethylene glycol, polypropylene glycol, polyoxyethylated glycerol, dextran, colonic acids, poly β -amino acids, and carbohydrate polymers.

20. The substantially purified compound of claim 19
30 wherein X is polyethylene glycol.

21. The substantially purified compound of claim 1
wherein said peptidic binding groups R_1 and R_2 are covalently bonded to said non-peptidic polymeric spacer
35 by thio-ether bonds.

22. The substantially purified compound of claim 21

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wherein cysteine residues of said peptidic binding groups R_1 and R_2 are part of said thio-ether bond.

23. The substantially purified compound of claim 1
5 wherein said polypeptidic binding groups are attached to said non-peptidic polymeric spacer via a cysteine residue.

24. The substantially purified compound of claim 23
10 wherein said cysteine is not native to the naturally occurring polypeptidic binding group.

25. The substantially purified compound of claim 1
15 wherein said compound has biological properties distinct from those of R_1 and R_2 alone.

26. The substantially purified compound of claim 2
wherein R_1 and R_2 are receptors.

27. The substantially purified compound of claim 2
20 wherein R_1 and R_2 are receptor antagonists.

28. The substantially purified compound of claim 2
25 wherein R_1 and R_2 are binding proteins.

29. The substantially purified compound of claim 2
wherein R_1 and R_2 are selected from the group
consisting of receptor antagonists, binding proteins
and receptors.

30. The substantially purified compound of claim 1
wherein R_1 and R_2 are biologically active portions of
proteins selected from the group consisting of:
interleukin-1 receptor antagonist, 30kDA tumor necrosis
35 factor inhibitor, IL-2 receptor, and CR1.

31. A substantially purified compound of the

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formula R_1-X-R_2 wherein:

R_1 and R_2 are polypeptidic groups that are proteins or biologically-active portions of proteins selected from the group consisting of interleukin-1 receptor antagonist, 30kDa tumor necrosis factor inhibitor, IL-2 receptors and CR1;

X is a non-peptidic polymer spacer selected from the group consisting of: polyethylene glycol, polypropylene glycol, polyoxyethylated glycerol, dextran, colonic acids, poly β -amino acids, and carbohydrate polymers, and

R_1 and R_2 are covalently attached to X by thio-ether bonds.

32. A pharmaceutical composition comprised of an effective amount of the substantially purified compound of claim 1 in a pharmacologically acceptable carrier.

33. A method for treating medical indications wherein patients in need thereof are administered the pharmaceutical composition of claim 32.

34. A method for the preparation of substantially purified therapeutically-valuable compounds, comprised of the formula R_1-X-R_2 , wherein R_1 and R_2 are cysteine-containing polypeptidic groups and X is a non-peptidic polymeric species comprised of:

reacting a non-peptidic polymeric group having at least two reactive groups capable of forming thio-ether bonds when reacted with cysteine amino acid residues with a cysteine containing polypeptidic group; isolating and purifying said compound.

35. The method of claim 34 wherein said polypeptidic group is selected from the group consisting of: interleukin-1 receptor antagonist, 30kDa tumor necrosis factor inhibitor, interleukin-2

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receptor, and CR1.

36. The method of claim 34 wherein said non-peptidic polymeric group is comprised of a polymeric unit selected from the group consisting of:
5 polyethylene glycol, polypropylene glycol, polyoxethylated glycerol, dextran, colonic acid, poly β -amino acids and carbohydrate polymers.

10 37. The method of claim 34 wherein said non-peptidic polymeric group is bis-maleimido polyethylene glycol.

38. A method for the preparation of substantially purified therapeutically valuable compounds comprised of the formula R_1-X-R_2 , wherein R_1 and R_2 are different, comprised of:

15
20 reacting a non-peptidic polymeric group having at least two reactive groups capable of forming thio-ether bonds when reacted with cysteine amino acid residues with a cysteine containing polypeptidic group R_1 to form a complex R_1-X ;

25 reacting complex R_1-X with a cysteine containing polypeptidic group R_2 to form said compound;
isolating and purifying said compound.

39. The method of claim 38 wherein R_1 and R_2 are selected from group consisting of: interleukin-2 receptor antagonist, 30kDa tumor necrosis factor inhibitor, interleukin-2 receptor, CR-1.
30

40. The method of claim 38 wherein said non-peptidic polymeric group is comprised of a polymeric unit selected from the group consisting of:
35 polyethylene glycol, polypropylene glycol, polyoxethylated glycerol, dextran, colonic acid, poly β -amino acids, and carbohydrate polymers.

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41. The method of claim 38 wherein said non-peptidic polymeric group is bismaleimide polyethylene glycol.
- 5 42. A substantially purified compound prepared by the method of claim 34.
43. A substantially purified compound prepared by the method of claim 38.
- 10 44. Native interleukin-1 receptor antagonist wherein cysteine residue 116 is covalently attached to a non-peptidic polymer.
- 15 45. The native interleukin-1 receptor antagonist of claim 44 wherein said polymer is monomethoxy polyethylene glycol.
- 20 46. The native interleukin-1 receptor antagonist of claim 44 wherein two native interleukin-1 receptor antagonists are bound to said non-peptidic polymer.
- 25 47. A mutein of interleukin-1 receptor antagonist wherein native interleukin-1 receptor antagonist is modified to contain at least one non-native cysteine residue.
- 30 48. The mutein of claim 47 wherein said non-native cysteines are found at amino acid residue sites selected from the group consisting of 0, 84, 6, 8, 9 and 141.
- 35 49. The mutein of claim 47 wherein the cysteine at position 116 of native interleukin-1 receptor antagonist is replaced with another amino acid residue.
50. The mutein of claim 47 wherein at least one

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cysteine residue is covalently attached to non-peptidic polymer.

51. The mutein of claim 49 wherein at least one of
5 said non-native cysteine residues is covalently
attached to a non-peptidic polymer.

52. The mutein of claim 47 wherein two muteins are
bound to a non-peptidic polymer.

10 53. The mutein of claim 48 wherein two muteins are
bound to a non-peptidic polymer.

54. A mutein of 30kDa tumor necrosis factor
15 inhibitor wherein native 30kDa tumor necrosis factor
inhibitor is modified to contain at least one non-
native cysteine residue.

55. The mutein of claim 54 wherein said non-native
20 cysteine is found at amino acid residue sites selected
from the group consisting of 1, 14, 105, 111 and 165.

56. The mutein of claim 54 wherein at least one of
said non-native cysteine residues is covalently
25 attached to a non-peptidic polymer.

57. The mutein of claim 54 wherein two muteins are
bound to a non-peptidic polymer.

30 58. A method for the preparation of
therapeutically- valuable polypeptides having an
increased apparent molecular weight comprised of:
altering the gene coding for said polypeptide
by site directed mutagenesis to create a gene coding
35 for a mutein of said polypeptide containing at least
one
non-native cysteine residue;

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expressing said altered gene in a bacterial expression system;

purifying said expressed mutein;

5 refolding said mutein in the presence of a sulfhydryl-containing compound;

reducing said refolded mutein with a mild reducing agent to free said non-native cysteines; and

10 reacting said mutein with a non-peptidic polymer group containing an activating group that is sulfhydryl specific.

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1	R	P	S	G	R	K	S	S	K	M	Q	A	F	R	I
16	W	D	V	N	Q	T	F	Y	L	R	N	N	Q	L	V
36	Q	G	P	N	V	N	L	E	E	K	I	D	V	V	P
56	L	F	L	G	I	H	G	G	K	M	C	L	S	C	V
76	T	R	L	Q	L	E	A	V	N	I	T	D	L	S	E
96	K	R	F	A	F	I	R	S	D	S	G	P	T	T	S
116	C	P	G	W	F	L	C	T	A	M	E	A	D	Q	P
	M	P	D	E	G	V	M	V	T	K	F	Y	F	Q	E

FIG.1

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10 Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Ser Ile Cys Cys Thr
 20
 30 Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
 40
 50 Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu Arg His Cys Leu
 60
 70 Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
 80
 90 Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu
 100
 110 Phe Gln Cys Phe Asn Cys Ser Leu Cys Ser Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
 120
 130 Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
 140
 150 Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu
 160
 161 Asn

FIG.2

SUBSTITUTE SHEET

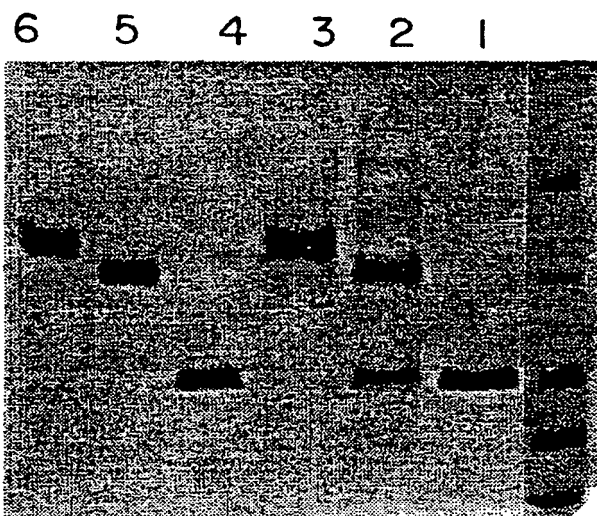


FIG.3

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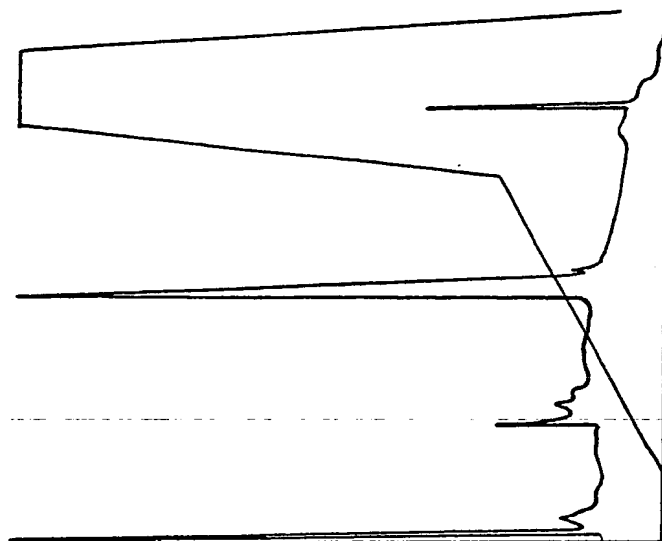


FIG. 4B

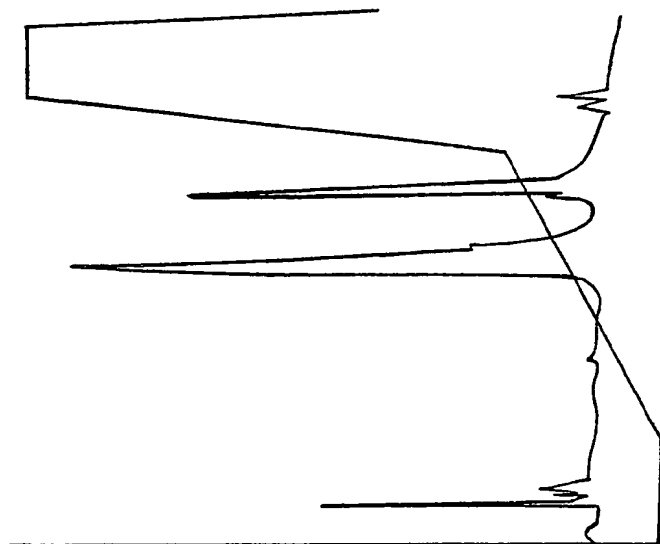


FIG. 4A

SUBSTITUTE SHEET

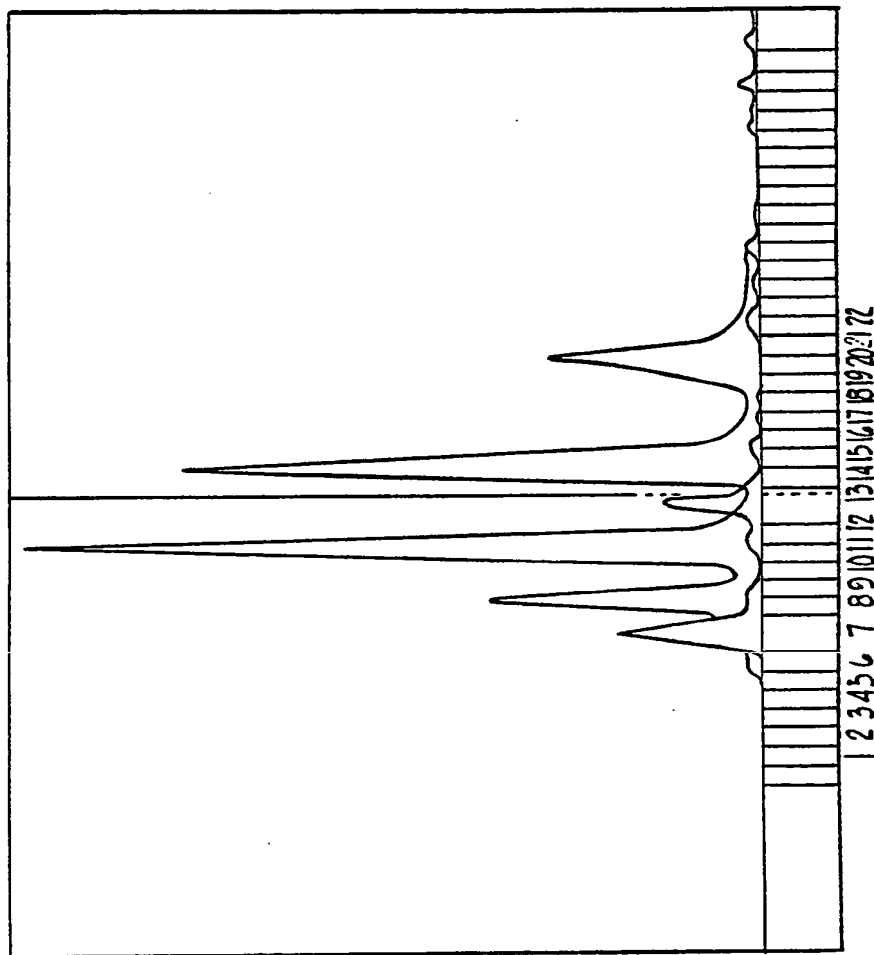


FIG. 5

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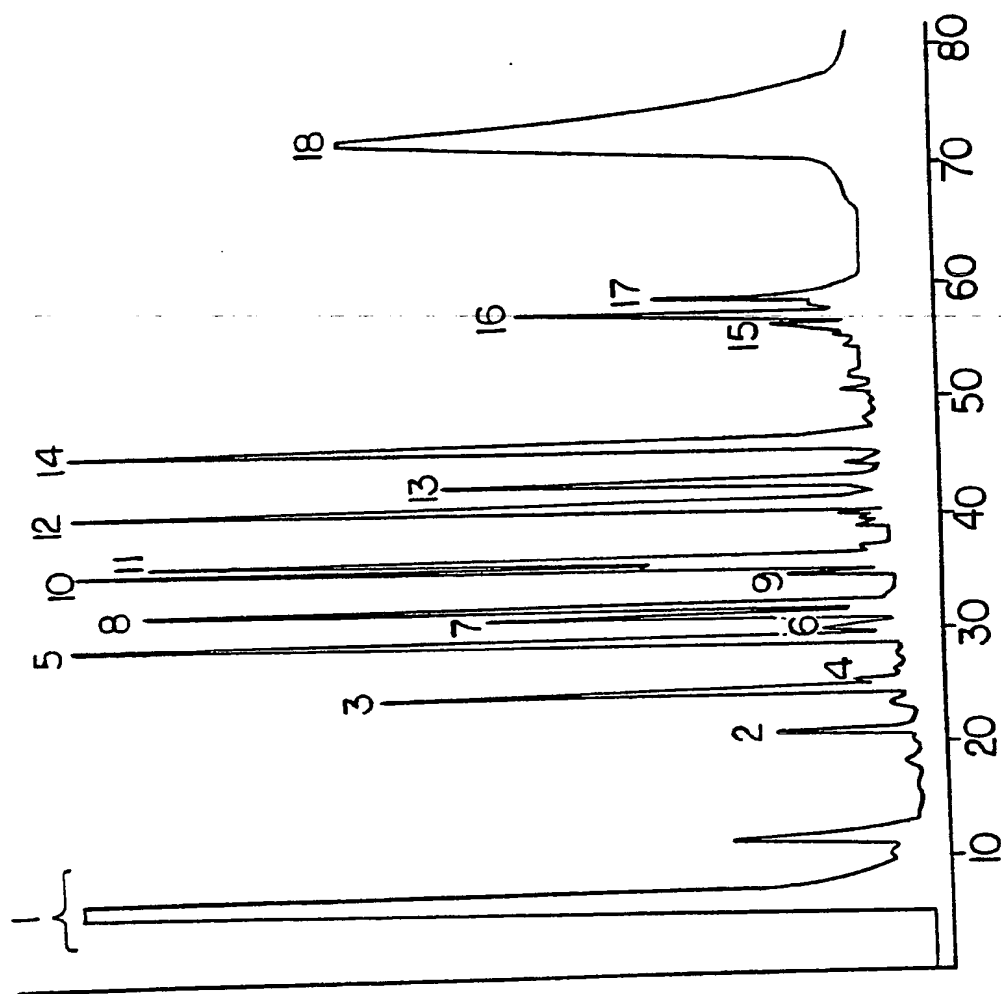


FIG. 6

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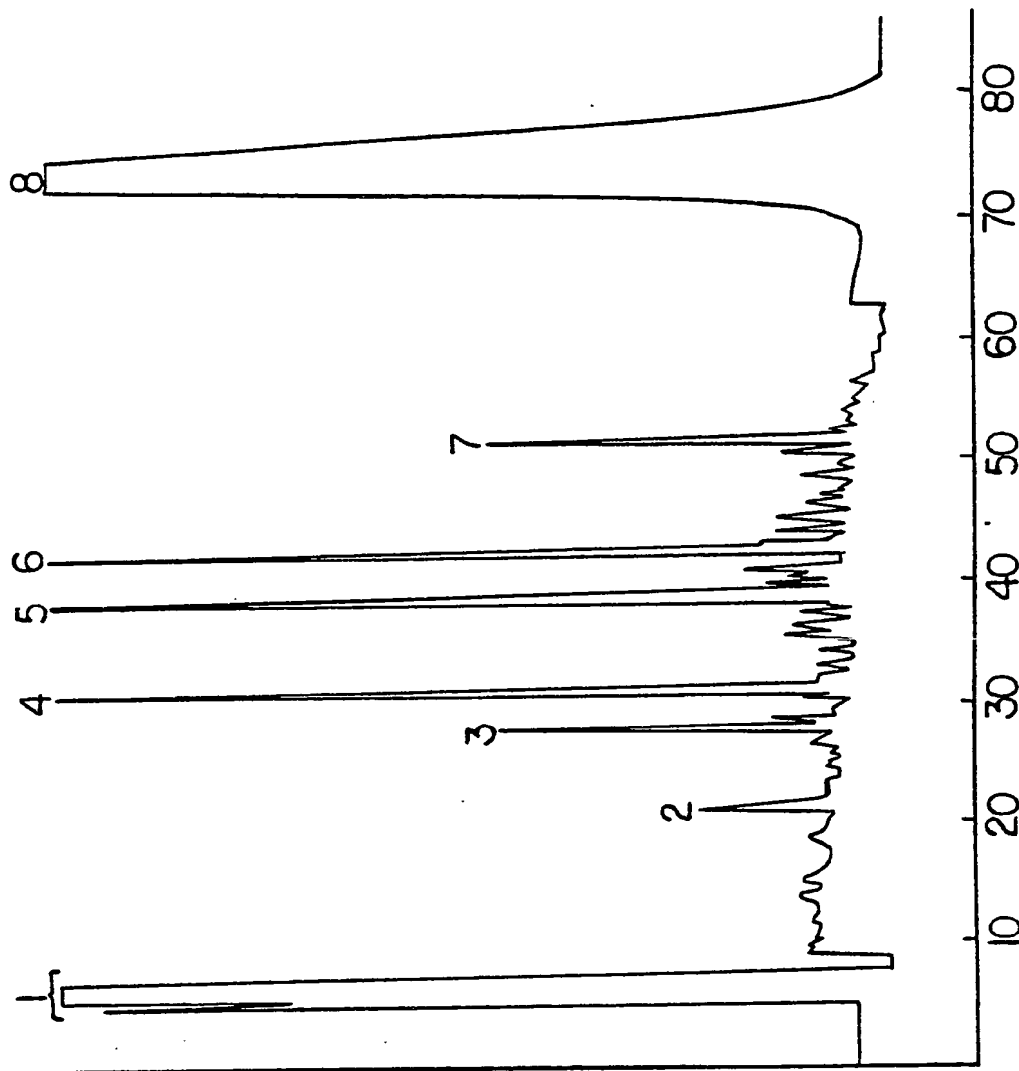


FIG. 7

SUBSTITUTE SHEET

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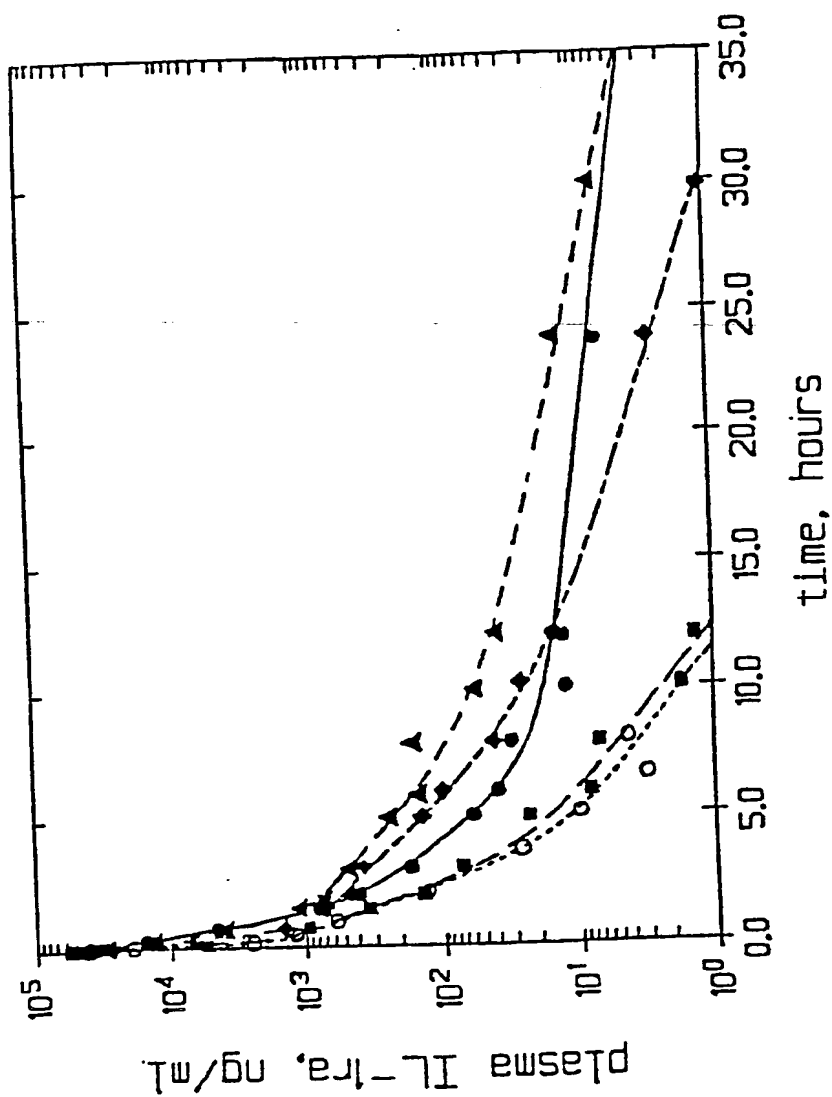


FIG.8

SUBSTITUTE SHEET

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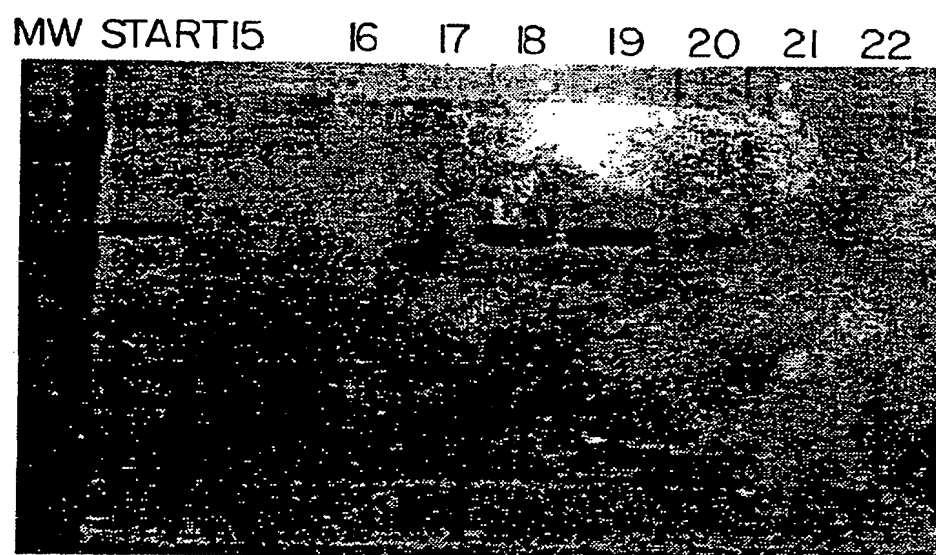


FIG. 9

SUBSTITUTE SHEET

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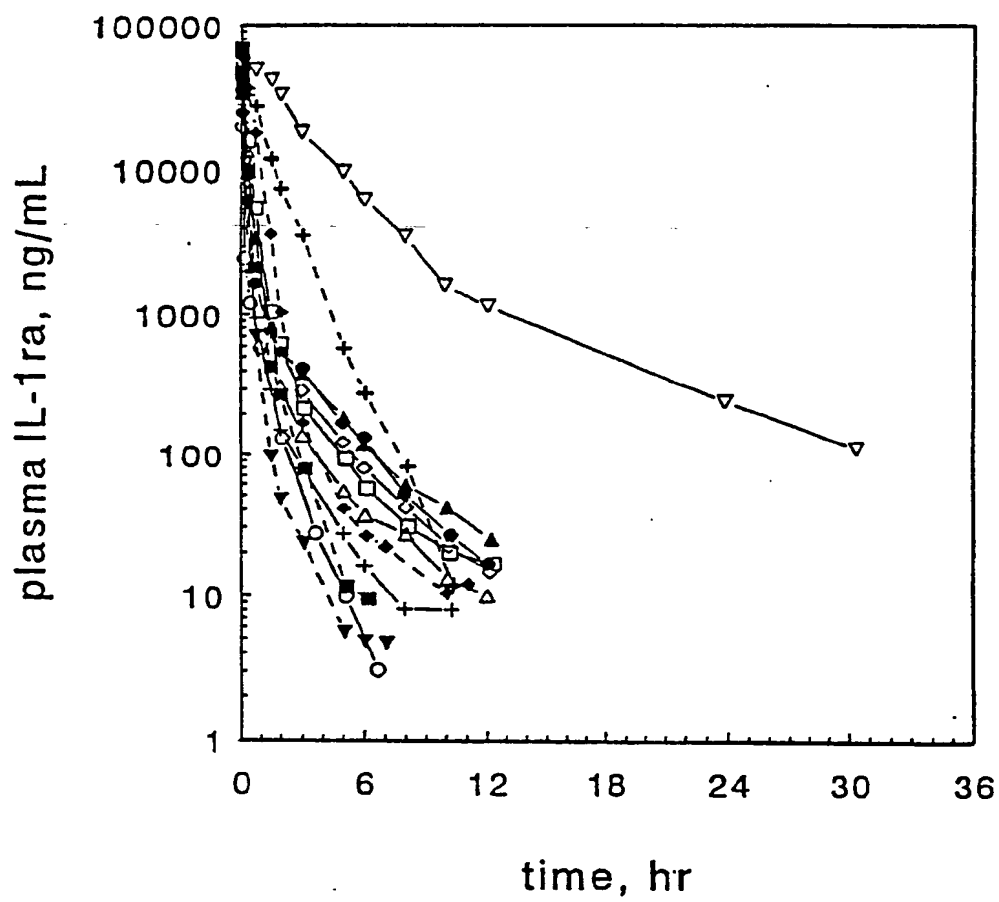


FIG.10

SUBSTITUTE SHEET

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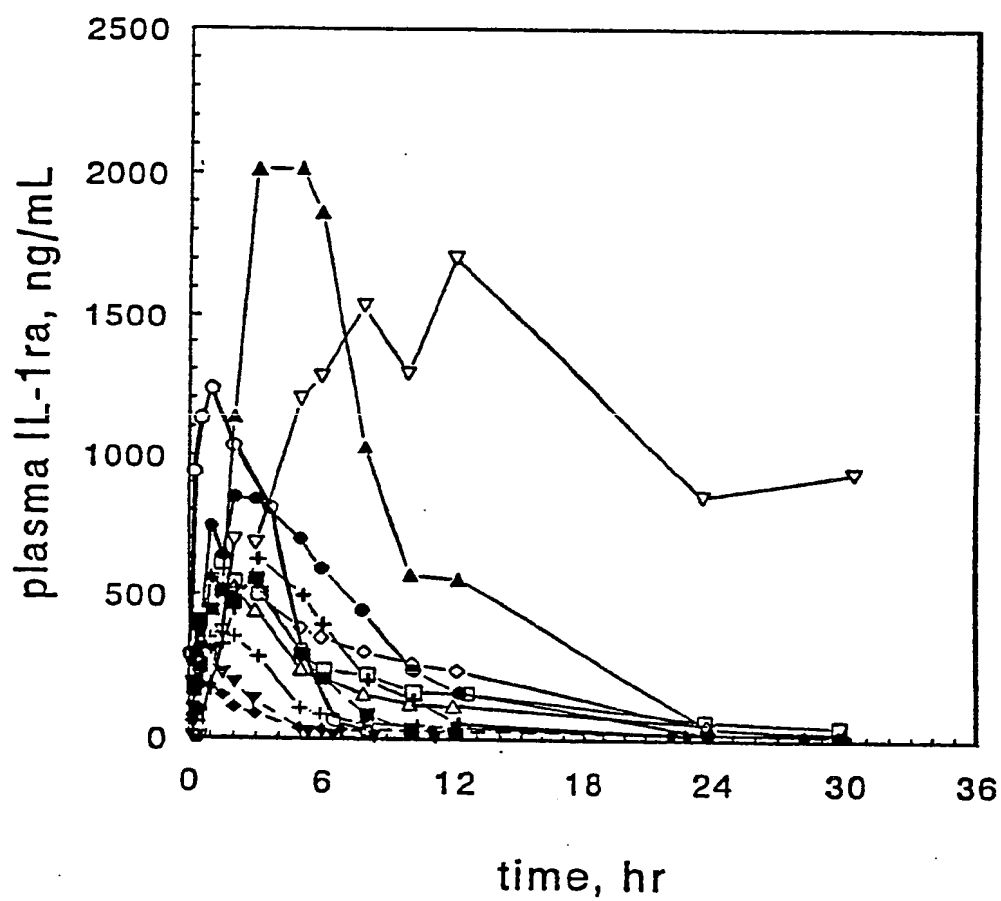


FIG. II

SUBSTITUTE SHEET

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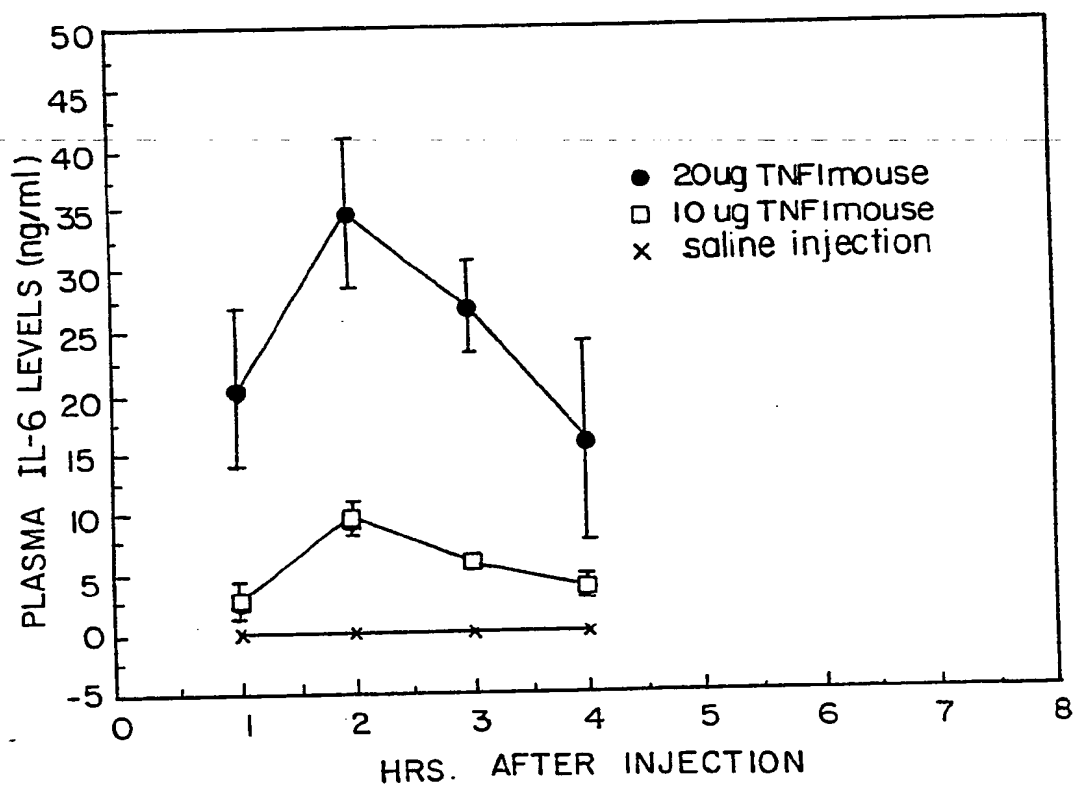


FIG.12

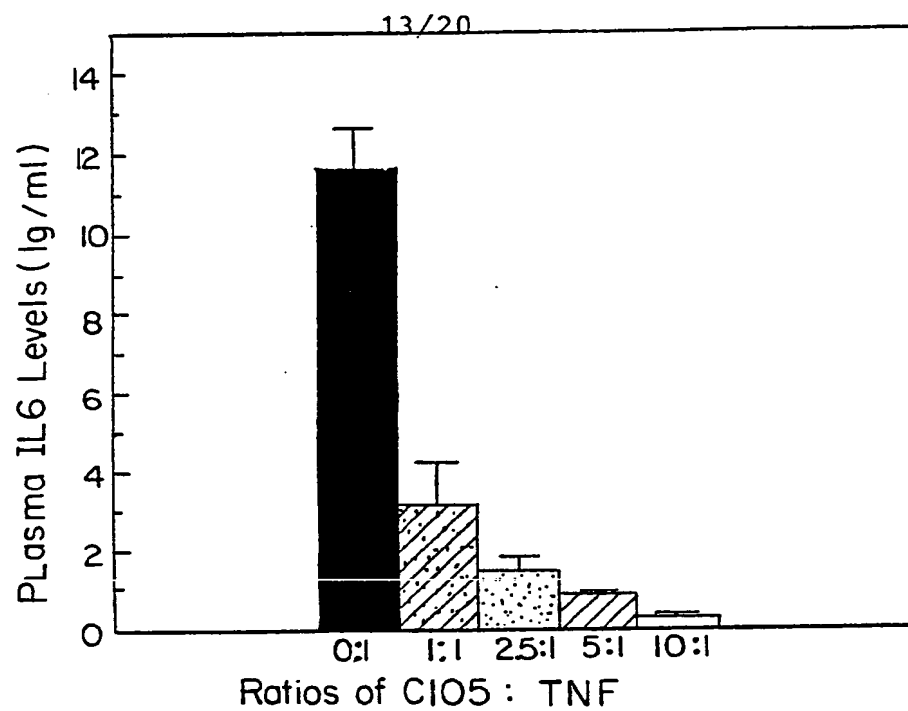


FIG.13 A

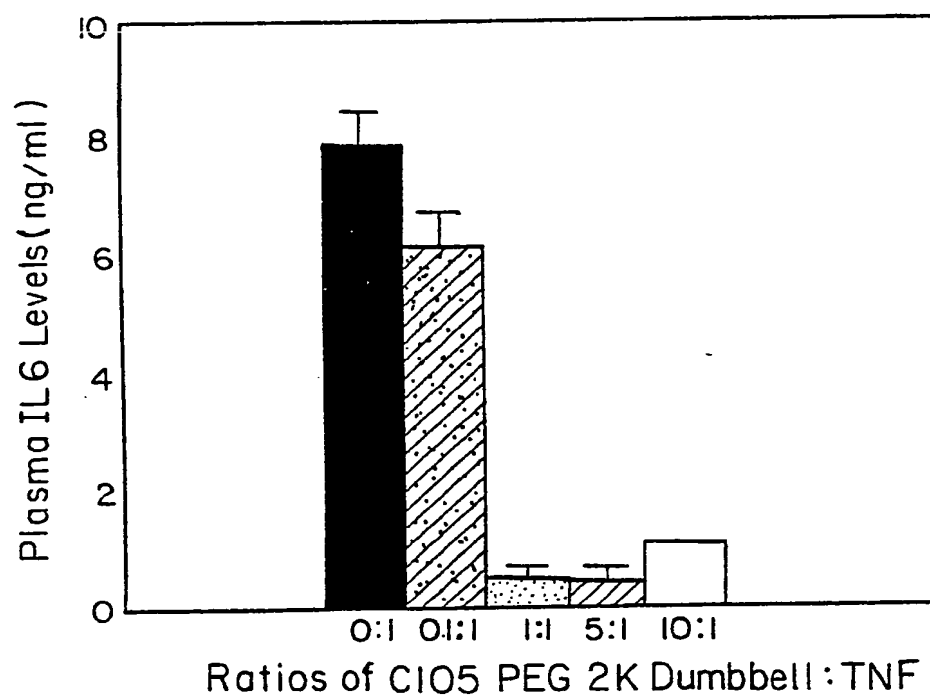


FIG.13 B

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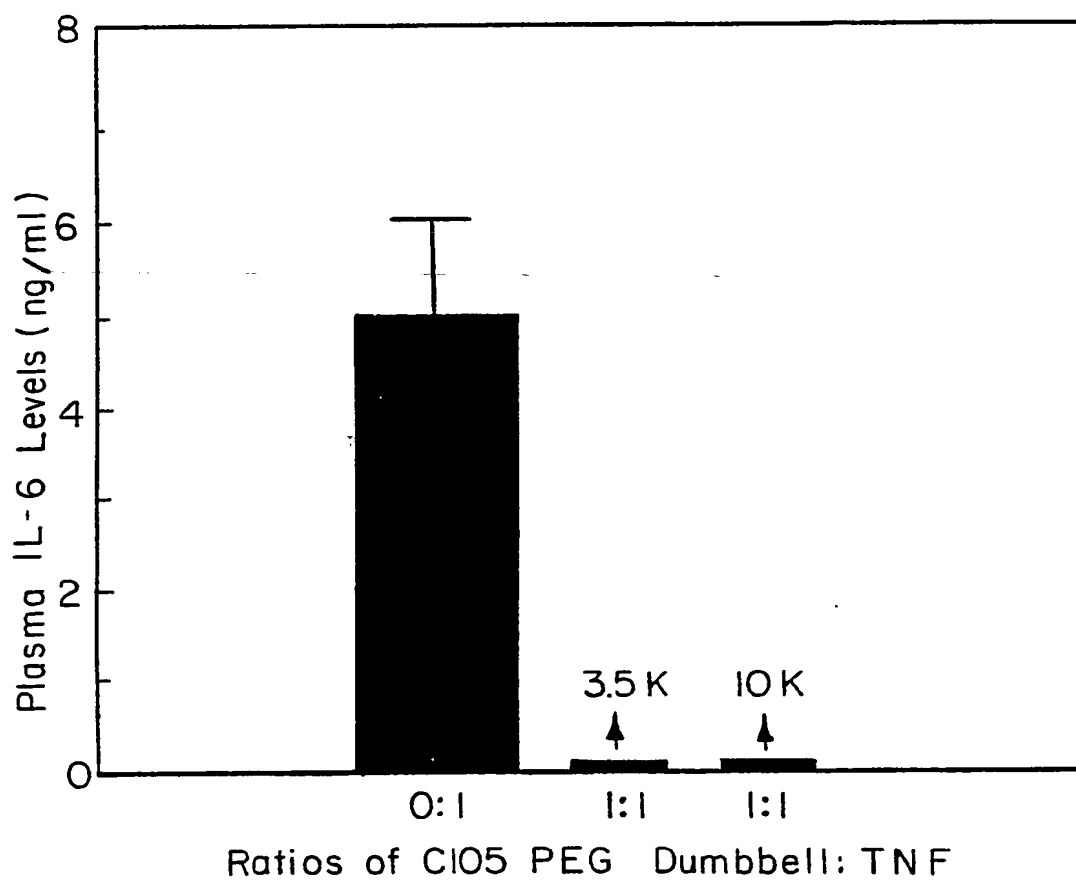


FIG.14

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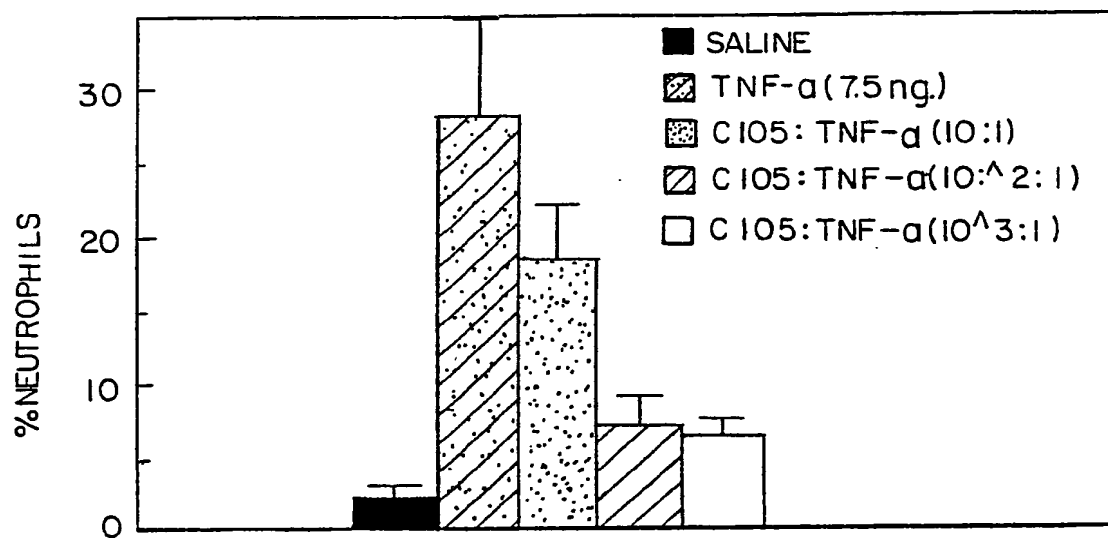


FIG 15A

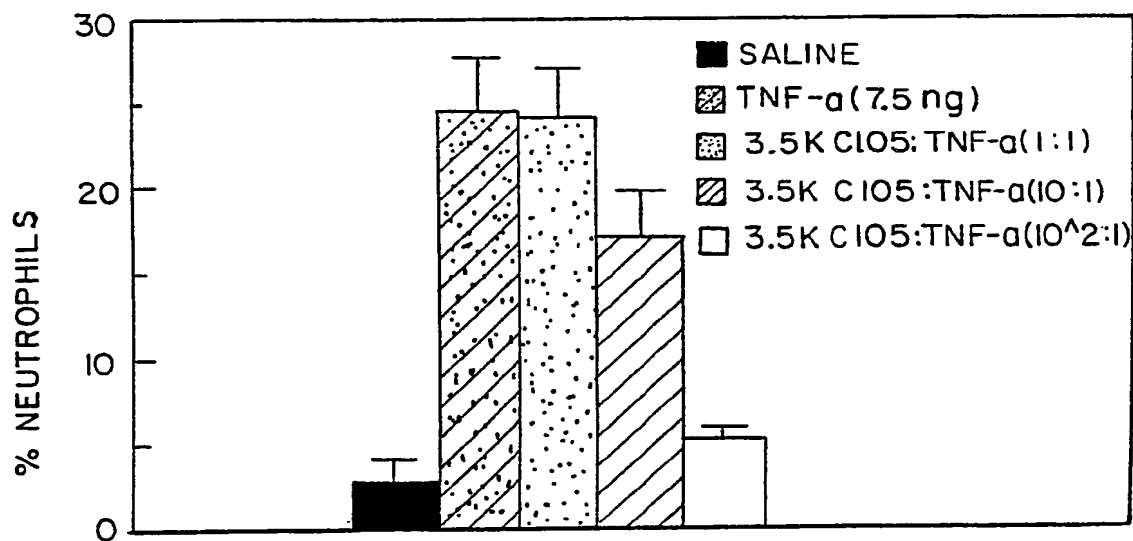


FIG.15B

SUBSTITUTE SHEET

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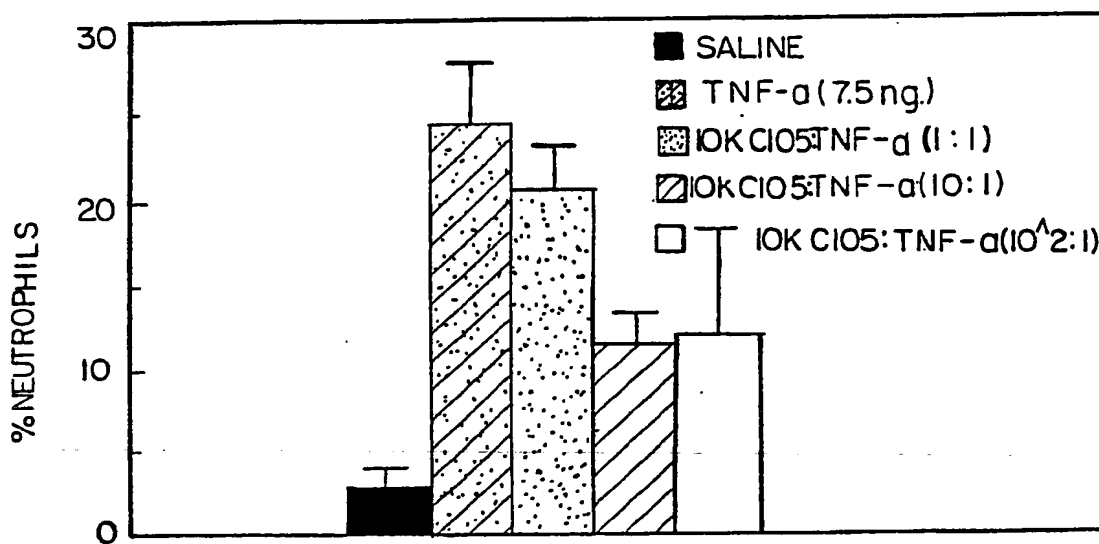


FIG 15C

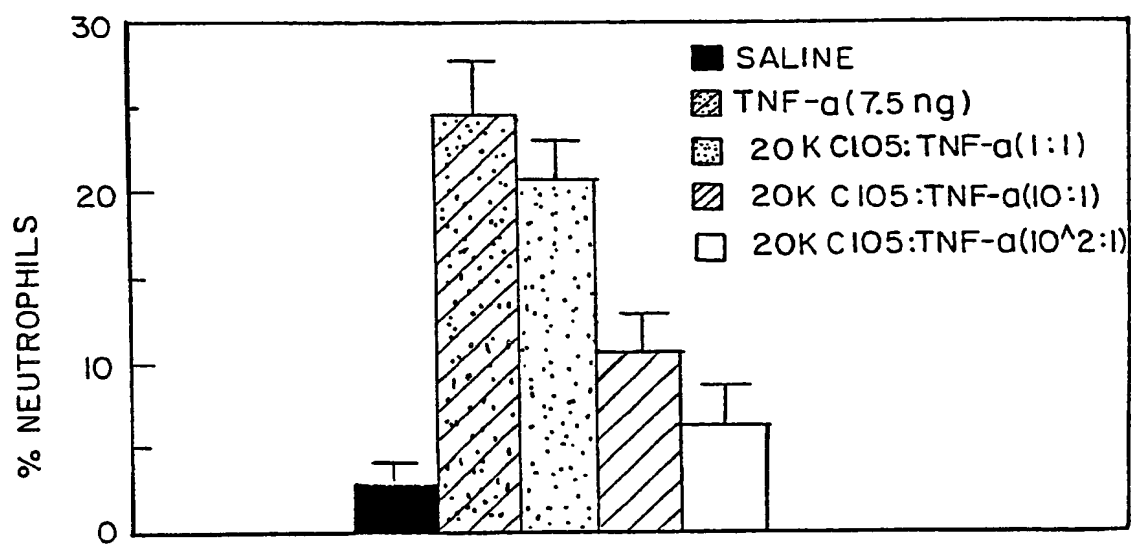


FIG.15D

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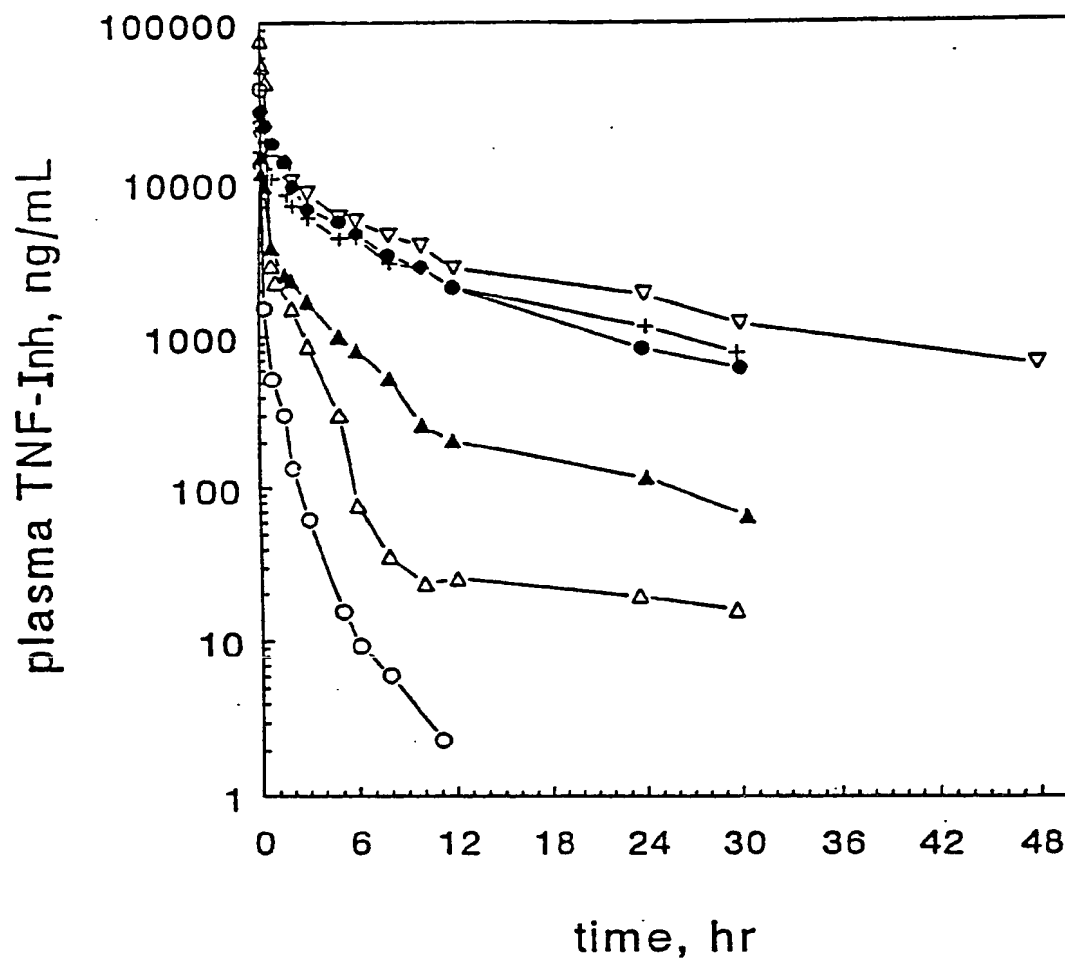


FIG.16

SUBSTITUTE SHEET

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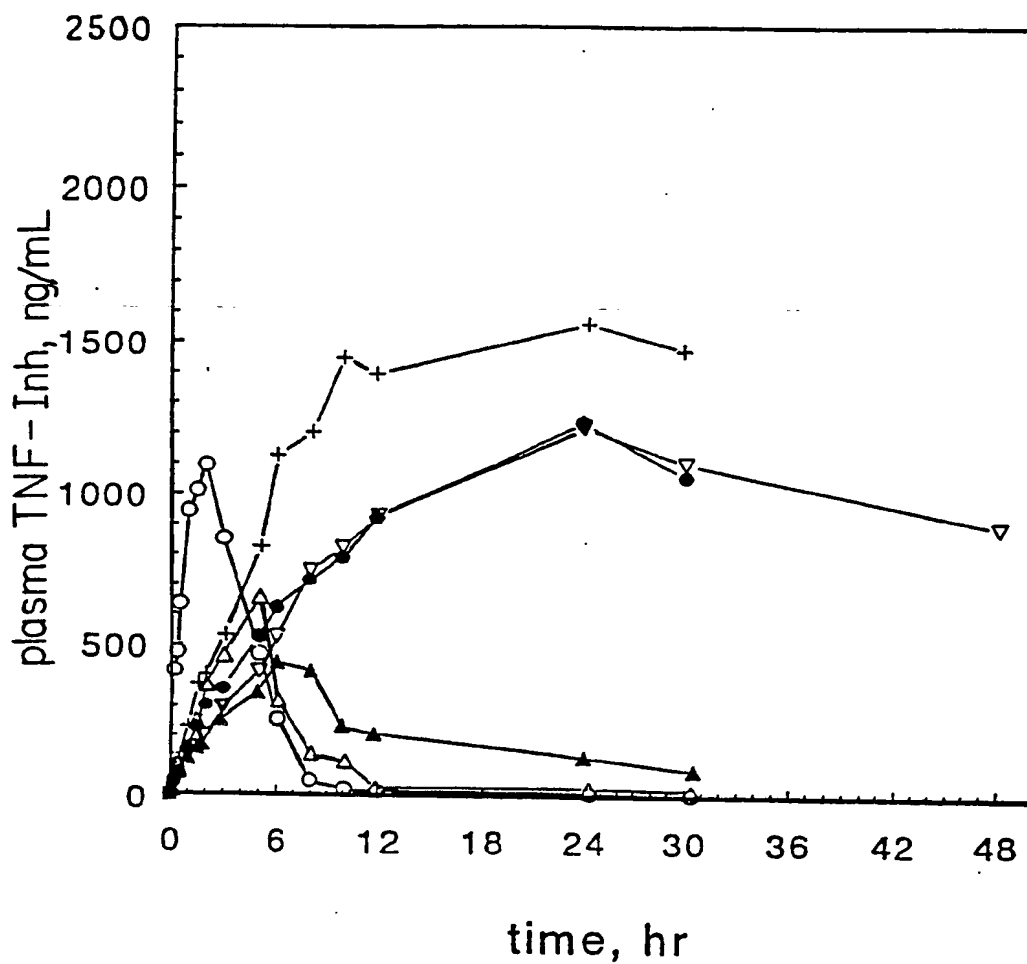


FIG.17

SUBSTITUTE SHEET

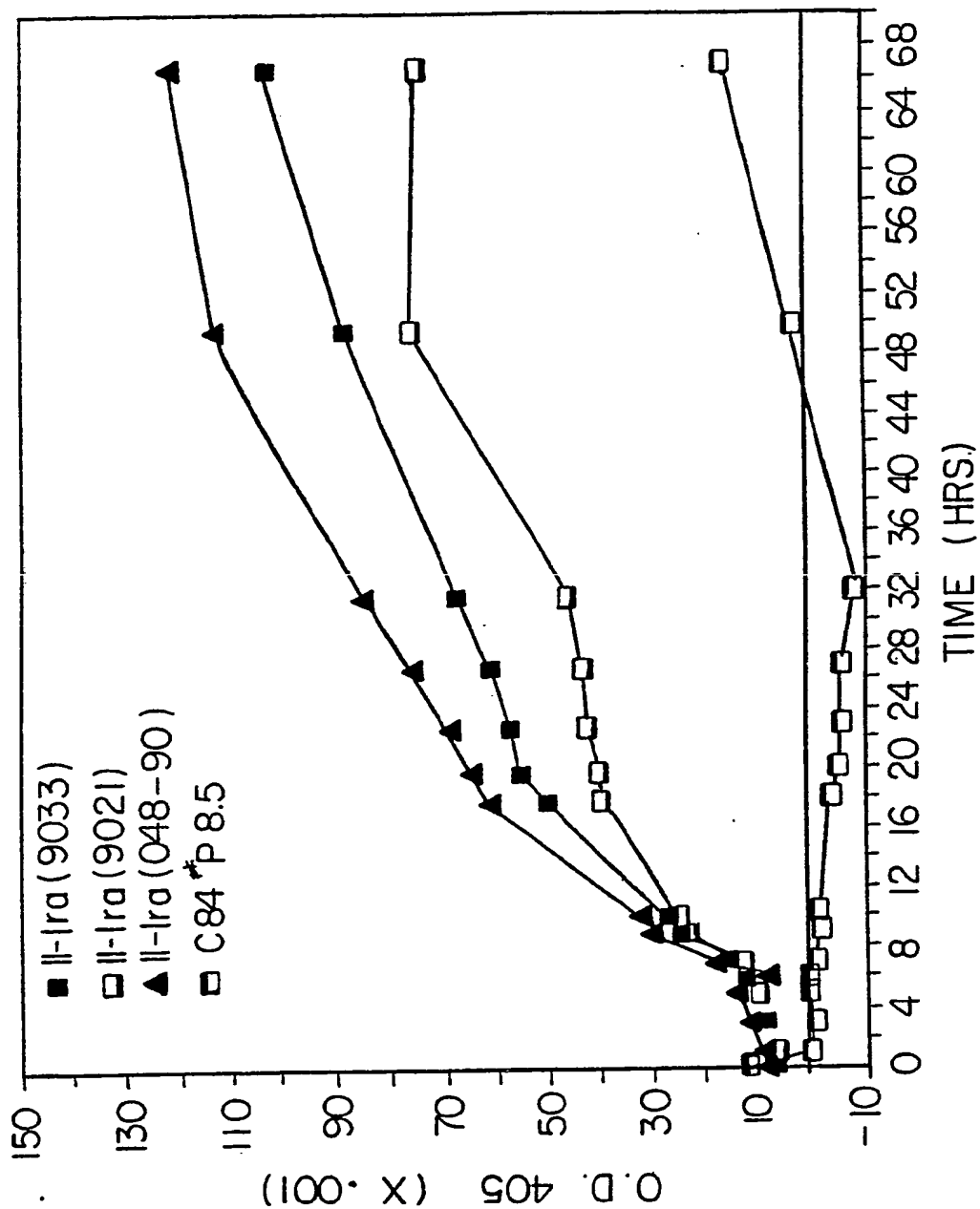


FIG.18

SUBSTITUTE SHEET

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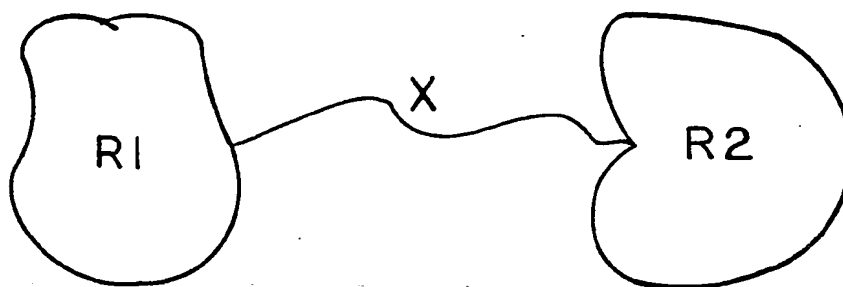


FIG.19

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02122

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 37/00; C07K 3/00, 13/00, 15/00 US CL : 514/12; 435/69.7, 240.2; 530/410, 395, 399																							
II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; padding: 2px;">Classification System</th> <th style="border: 1px solid black; padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; padding: 5px;">U.S.</td> <td style="border: 1px solid black; padding: 5px;">514/12; 435/69.7, 240.2; 530/410, 395, 399</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched⁵</div>			Classification System	Classification Symbols	U.S.	514/12; 435/69.7, 240.2; 530/410, 395, 399																	
Classification System	Classification Symbols																						
U.S.	514/12; 435/69.7, 240.2; 530/410, 395, 399																						
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category⁶</th> <th style="width: 70%; padding: 2px;">Citation of Document,¹⁶ with indication, where appropriate, of the relevant passages¹⁷</th> <th style="width: 20%; padding: 2px;">Relevant to Claim No. ¹⁸</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 2px;">Y</td> <td style="padding: 2px;">US, A, 4,578,335 (Urdal et al.) 25 March 1986, see entire document</td> <td style="text-align: center; vertical-align: top; padding: 2px;">4, 5, 12 - 14, 17, 18, 26, 28 - 31, 35, 39</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 2px;">Y</td> <td style="padding: 2px;">US, A, 4,789,658 (Yoshimoto et al.) 06 December 1988, see entire document</td> <td style="text-align: center; vertical-align: top; padding: 2px;">4, 17</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 2px;">Y</td> <td style="padding: 2px;">Cell, Vol. 61, issued 20 April 1990, Schall et al, "Molecular cloning and expression of a receptor for human tumor necrosis factor", pages 361-370, see entire document.</td> <td style="text-align: center; vertical-align: top; padding: 2px;">26, 29</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 2px;">Y</td> <td style="padding: 2px;">Proceedings of the National Academy of Sciences, Vol. 87, Issued October 1990, Gray et al., "Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein", pages 7380-7384, see entire document.</td> <td style="text-align: center; vertical-align: top; padding: 2px;">26, 28, 29</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 2px;">Y</td> <td style="padding: 2px;">E. Goldberg et al., "BIOMEDICAL POLYMERS: POLYMERIC MATERIALS AND PHARMACEUTICALS FOR BIOMEDICAL USE", published 1980 by Academic Press (N.Y.), see pages 441-452.</td> <td style="text-align: center; vertical-align: top; padding: 2px;">1-57</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 2px;">Y</td> <td style="padding: 2px;">US, A, 4,847,325 (Shadle et al) 11 July 1989, see entire document.</td> <td style="text-align: center; vertical-align: top; padding: 2px;">1-57</td> </tr> </tbody> </table>			Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	Y	US, A, 4,578,335 (Urdal et al.) 25 March 1986, see entire document	4, 5, 12 - 14, 17, 18, 26, 28 - 31, 35, 39	Y	US, A, 4,789,658 (Yoshimoto et al.) 06 December 1988, see entire document	4, 17	Y	Cell, Vol. 61, issued 20 April 1990, Schall et al, "Molecular cloning and expression of a receptor for human tumor necrosis factor", pages 361-370, see entire document.	26, 29	Y	Proceedings of the National Academy of Sciences, Vol. 87, Issued October 1990, Gray et al., "Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein", pages 7380-7384, see entire document.	26, 28, 29	Y	E. Goldberg et al., "BIOMEDICAL POLYMERS: POLYMERIC MATERIALS AND PHARMACEUTICALS FOR BIOMEDICAL USE", published 1980 by Academic Press (N.Y.), see pages 441-452.	1-57	Y	US, A, 4,847,325 (Shadle et al) 11 July 1989, see entire document.	1-57
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p> </div> </div>																							
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search² <div style="text-align: center; font-weight: bold;">02 June 1992</div> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report² <div style="text-align: center; font-size: 1.2em; font-weight: bold;">18 JUN 1992</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority¹ <div style="text-align: center; font-weight: bold;">ISA/US</div> </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer²⁰ <div style="text-align: center;"> LORRAINE M. SPECTOR, PH.D. </div> </td> </tr> </table>			Date of the Actual Completion of the International Search ² <div style="text-align: center; font-weight: bold;">02 June 1992</div>	Date of Mailing of this International Search Report ² <div style="text-align: center; font-size: 1.2em; font-weight: bold;">18 JUN 1992</div>	International Searching Authority ¹ <div style="text-align: center; font-weight: bold;">ISA/US</div>	Signature of Authorized Officer ²⁰ <div style="text-align: center;"> LORRAINE M. SPECTOR, PH.D. </div>																	
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US, A, 4,766,106 (Katre et al) 23 August 1988, see entire document especially abstract, col. 1, col. 3, lines 57-62, col 4.	1-57
Y	US, A, 4,935,233 (Bell et al.) 19 June 1990, see entire document.	1-57

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. ☐ Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:
2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:
3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:
Please See Attached Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
1-57 (Telephone Practice) (Telephone Practice)
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-57, drawn to pegylated proteins, method of making same and use thereof, Class 514, subclass 12, Class 435 subclasses 69.7 and 240.2, and Class 530, subclasses 410, 395 and 399.

II. Claim 58, drawn to a method of making muteins, Class 435, subclasses 172.1 and 71.2, and Class 530, subclass 402.

The claims of groups I and II are drawn to distinct methods and have a separate status in the art as shown by their different classification. Group I is drawn to the chemical linkage of proteins, whereas Group II is drawn to site-directed mutagenesis for the production of muteins. PCT Rules 13.1 and 13.2 do not provide for multiple distinct methods within a single general inventive concept.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
19 June 2003 (19.06.2003)

PCT

(10) International Publication Number
WO 03/049684 A2

(51) International Patent Classification⁷: **A61K**

(21) International Application Number: PCT/US02/38839

(22) International Filing Date: 4 December 2002 (04.12.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/336,707 7 December 2001 (07.12.2001) US

(71) Applicant (*for all designated States except US*): **CENTOCOR, INC.** [US/US]; 200 Great Valley Parkway, Malvern, PA 19355 (US).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **HEAVNER, George** [US/US]; 6 Oak Glen Drive, Malvern, PA 19355 (US).

(74) Agents: **JOHNSON, Philip, S. et al.**; One Johnson & Johnson Plaza, New Brunswick, NJ 08933 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 03/049684 A2

(54) Title: **PSEUDO-ANTIBODY CONSTRUCTS**

(57) Abstract: This invention relates to novel pharmaceutically useful compositions that bind to a biological molecule, having improved circulatory half-life, increased avidity, increased affinity, or multifunctionality, and methods of use thereof. The present invention provides a pseudo-antibody comprising an organic moiety covalently coupled to at least two target-binding moieties, wherein the target-binding moieties are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule. The pseudo-antibody of the present invention may affect a specific ligand *in vitro*, *in situ* and/or *in vivo*. The pseudo-antibodies of the present invention can be used to measure or effect in an cell, tissue, organ or animal (including humans), to diagnose, monitor, modulate, treat, alleviate, help prevent the incidence of, or reduce the symptoms of, at least one condition.

PSEUDO-ANTIBODY CONSTRUCTS

This application claims priority to US provisional application 60/336,707, filed
5 December 7, 2001, and which application is entirely incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to novel pharmaceutically useful compositions that bind
to a biological molecule, having improved circulatory half-life, increased avidity,
10 increased affinity, or multifunctionality, and methods of use thereof.

BACKGROUND OF THE INVENTION

Numerous pharmaceutical compounds and peptides have been identified that
bind to a biological molecule and that affect biological activity. Recombinant protein
15 technology has provided numerous promising therapeutic agents. Advances in protein
formulation and chemical modification of these therapeutic proteins have lead to
improved resistance to proteolytic enzymes and decreased immunogenicity, thus
increasing the therapeutic protein's stability, circulatory half-life, and
biological activity.

20 Antibodies provide an example of recombinant proteins with great therapeutic
potential. Full antibodies are bivalent molecules composed of two identical Fab
domains and an Fc domain. The Fab domains contain two identical binding sites,
sometimes referred to as paratopes, each within the variable regions at the N-termini of
the Fab domains, and comprised of complementarity determining regions (CDRs).
25 Antibodies have additional functionality in their Fc domains, that can offer additional
functionality beyond the binding of the CDRs in the variable regions. There are
instances, however, when Fc-mediated activity can be disadvantageous. For example,
an antibody fragment that binds to the GPIIb/IIIa receptors on platelets can block
platelet aggregation, but the presence of an Fc domain would result in platelet clearance
30 and thrombocytopenia. Antibodies can be subjected to proteolysis to remove the Fc
domain, creating either Fab or Fab'₂ fragments. These non-glycosylated antibody
fragments have molecular weights of approximately 50,000 and 100,000 where the

parent antibodies have molecular weights of approximately 150,000 and can be glycosylated. And although antibody fragments may be advantageous therapeutically, antibody fragments are generally cleared at a faster rate than the intact antibodies. Capon et al., 337 NATURE 525-31 (1989).

5 A limited number of constructs have been prepared where the Fab domains have been modified. In particular, synthetic moieties such as PEG have been added to the Fab to increase the molecular weight and slow down clearance. *See, e.g.*, WO 00/26256; published May 11, 2000.

 Antibodies, proteins, and peptides have been modified with polyethyleneglycol
10 (PEG) to increase half-life, decrease degradation and decrease immunogenicity. Derivatized PEG compounds have been discussed previously. *See* U.S. Pat. No. 5,438,040.

 Yet, there remains a need in the field for improved modified therapeutic
antibodies. More specifically, these modifications, as described herein, improve the
15 pharmacokinetic properties (e.g., increase *in vivo* serum half-life) without significantly affecting the antigen-binding properties (e.g., affinity) of the antigen-binding moieties, while potentially increasing avidity and providing, for example, a single pseudo-antibody that binds more than one type of antigen or receptor. This invention thus
provides for the construction of entirely new families of pseudo-antibodies (Ψ Ab) using either Fab or Fab' fragments prepared from antibodies, single chain antibodies
20 (sFv), peptides that bind to proteins or other biological molecules, or organic compounds that bind to proteins or other biological molecules.

SUMMARY OF INVENTION

25 The present invention provides a pseudo-antibody comprising an organic moiety covalently coupled to two or more identical target-binding moieties, wherein said target-binding moieties are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule. The present invention also provides for a pseudo-antibody
30 comprising an organic moiety covalently coupled to two or more different target-binding moieties, wherein said target-binding moieties are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule.

The pseudo-antibody of the present invention may affect a specific ligand, such as where the pseudo-antibody modulates, decreases, increases, antagonizes, angonizes, mitigates, alleviates, blocks, inhibits, abrogates and/or interferes with at least one biological molecule's activity or binding, or with a receptor activity or binding, *in vitro*, *in situ* and/or *in vivo*. The pseudo-antibodies of the present invention can be used to measure or effect in an cell, tissue, organ or animal (including humans), to diagnose, monitor, modulate, treat, alleviate, help prevent the incidence of, or reduce the symptoms of, at least one condition. The pseudo-antibody constructs may be used to treat stenosis and/or restenosis following a vascular intervention, to prevent ischemia, to inhibit the growth and/or metastasis of a tumor, to inhibit a biological process mediated by the binding of a ligand to either or both of GPIIb/IIIa and $\alpha_v\beta_3$, expressed on the plasma membrane of a cell, or to inhibit angiogenesis. Such a method can comprise administering an effective amount of a composition or a pharmaceutical composition comprising at least one pseudo-antibody to a cell, tissue, organ, animal or patient in need of such modulation, treatment, alleviation, prevention, or reduction in symptoms, effects or mechanisms. The effective amount can comprise an amount effective amount per single, multiple or continuous administration.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a comparison of the inhibition of platelet aggregation by two pseudo-antibodies (7E3 Fab'(PEG_{3.4K} - DSPE)₂ and 7E3 Fab'(PEG_{3.4K} - PAL)₂) and one unmodified antibody fragment (7E3 Fab).

Figure 2 depicts a comparison of the inhibition of platelet aggregation by two pseudo-antibodies (7E3 Fab'(PEG_{5K})₂ and 7E3 Fab'(PEG_{10K})₂) and one unmodified antibody fragment (ReoPro®).

Figure 3 depicts a comparison of *in vivo* circulating half-life, in mice, of two pseudo-antibodies, 7E3 Fab'(PEG_{3.4K} - DSPE)₂ and 7E3 Fab'(PEG_{5K})₂.

DETAILED DESCRIPTION

It is to be understood that this invention is not limited to the particular methodology, protocols, constructs, formulae and reagents described and as such may vary. It is also to be understood that the terminology used herein is for the purpose of

describing particular embodiments only, and is not intended to limit the scope of the present.

It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a gene" is a reference to one or more genes and includes equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications and patents mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the constructs and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventor is not entitled to antedate such disclosure by virtue of prior invention.

The present invention provides for entirely new families of pseudo-antibodies (ΨAbs) using peptides that bind to antigens, receptors, proteins or other biological molecules, either Fab or Fab' fragments prepared from antibodies, single chain antibodies (sFv), or organic compounds that bind to proteins or other biological molecules (target-binding moieties). The target-binding moieties may be peptides identified or produced by various methods known in the art. The method of obtaining these moieties, or the physical characteristics of these moieties, are not limitations of the invention. Preferred structures are those that bind to a biological molecule to block binding to another biological molecule or bind to a biological molecule to initiate a biological event. Some advantages of the invention described herein are that it presents molecules that bind to biomolecules and: (a) enhances their avidity (the functional combining strength of an target-binding moiety with its target, which is related to both the affinity of the reaction between the epitopes and the paratopes, and the valencies of the target-binding moiety and target); (b) provides multivalent constructs; (c) increases their circulating half-lives by increasing molecular size; (d) creates specific binding to

multiple compounds by a single molecule; and/or (e) allows the incorporation of lipids, fatty acids, carbohydrates, steroids, etc.; that can bind to molecules other than the primary biological molecules and affect distribution to specific locations (e.g., fatty acid adducts could bind to serum albumin to keep molecules in circulation or lipid adducts could be used to provide non-covalent attachment of constructs to lipid-coated stents).

The target-binding moiety of the pseudo-antibody may include an immunoglobulin, an integrin, an antigen, a growth factor, a cell cycle protein, a cytokine, a hormone, a neurotransmitter, a receptor or fusion protein thereof, a blood protein, an antimicrobial, or any fragment, or structural or functional analog thereof. In addition, the target itself may be an immunoglobulin, an integrin, an antigen, a growth factor, a cell cycle protein, a cytokine, a hormone, a neurotransmitter, a receptor or fusion protein thereof, a blood protein, an antimicrobial, or any fragment, or structural or functional analog thereof.

For example, in one embodiment of the invention, the target-binding moieties of the pseudo-antibody may be derived from human or non-human polyclonal or monoclonal antibodies. Specifically, these antibodies (immunoglobulins) may be isolated, recombinant and/or synthetic human, primate, rodent, mammalian, chimeric, humanized or CDR-grafted, antibodies and anti-idiotypic antibodies thereto. Such moieties can be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Additionally, these binding moieties can also be produced in a variety of truncated forms in which various portions of antibodies are joined together chemically by conventional techniques, or prepared as a contiguous protein using genetic engineering techniques. As used presently, an "antibody," "antibody fragment," "antibody variant," "Fab," and the like, include any protein- or peptide- containing molecule that comprises at least a portion of an immunoglobulin molecule, such as but not limited to at least one CDR of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof, or at least one portion of a receptor or binding protein, which can be incorporated into a pseudo-antibody of the present invention. Such antibody optionally further affects a specific ligand, such as but not limited to, where such antibody modulates, decreases, increases, antagonizes, agonizes, mitigates, alleviates, blocks,

inhibits, abrogates and/or interferes with at least one target activity or binding, or with receptor activity or binding, *in vitro*, *in situ* and/or *in vivo*.

In one embodiment of the invention, such antibodies, or functional equivalents thereof, may be "human," such that they are substantially non-immunogenic in humans.

5 These antibodies may be prepared through any of the methodologies described herein, including the use of transgenic animals, genetically engineered to express human antibody genes. For example, immunized transgenic mice (xenomice) that express either fully human antibodies, or human variable regions have been described. WO 96/34096, published Oct. 31, 1996. In the case of xenomice, the antibodies produced
10 include fully human antibodies and can be obtained from the animal directly (e.g., from serum), or from immortalized B-cells derived from the animal, or from the genes encoding the immunoglobulins with human variable regions can be recovered and expressed to obtain the antibodies directly or modified to obtain analogs of antibodies such as, for example, Fab or single chain Fv molecules. *Id.*

15 The term "antibody" is further intended to encompass antibodies, digestion fragments, specified portions and variants thereof, including antibody mimetics or comprising portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. The present invention thus encompasses antibody fragments
20 capable of binding to a biological molecule (such as an antigen or receptor) or portions thereof, including but not limited to Fab (e.g., by papain digestion), Fab' (e.g., by pepsin digestion and partial reduction) and F(ab')₂ (e.g., by pepsin digestion), facb (e.g., by plasmin digestion), pFc' (e.g., by pepsin or plasmin digestion), Fd (e.g., by pepsin digestion, partial reduction and reaggregation), Fv or scFv (e.g., by molecular
25 biology techniques) fragments. *See, e.g.,* CURRENT PROTOCOLS IN IMMUNOLOGY, (Colligan et al., eds., John Wiley & Sons, Inc., NY, 1994-2001).

As with antibodies, other peptide moieties that bind a particular target protein or other biological molecule (target-binding peptides) are encompassed by the pseudo-antibody disclosed herein. Such target-binding peptides may be isolated from tissues
30 and purified to homogeneity, or isolated from cells which contain the target-binding protein, and purified to homogeneity. Once isolated and purified, such target-binding peptides may be sequenced by well-known methods. From these amino acid sequences, DNA probes may be produced and used to obtain mRNA, from which

cDNA can be made and cloned by known methods. Other well-known methods for producing cDNA are known in the art and may effectively be used. In general, any target-binding peptide can be isolated from any cell or tissue expressing such proteins using a cDNA probe such as the probe described above, isolating mRNA and
5 transcribing the mRNA into cDNA. Thereafter, the protein can be produced by inserting the cDNA into an expression vector, such as a virus, plasmid, cosmid, or other vector, inserting the expression vector into a cell, proliferating the resulting cells, and isolating the expressed target-binding protein from the medium or from cell extract as described above. Alternatively, target-binding peptides may be chemically synthesized
10 using the sequence described above and an amino acid synthesizer, or manual synthesis using chemical conditions well known to form peptide bonds between selected amino acids. Analogues and fragments of target-binding proteins may be produced by chemically modification or by genetic engineering. These fragments and analogues may then be tested for target-binding activity using known methods. *See, e.g.*, U.S.
15 Patent No. 5,808,029 to Brockhaus et al., issued Sept. 15, 1998.

Alternatively, target-binding peptides, including antibodies, may be identified using various library screening techniques. For example, peptide library screening takes advantage of the fact that molecules of only "peptide" length (2 to 40 amino acids) can bind to the receptor protein of a given large protein ligand. Such peptides
20 may mimic the bioactivity of the large protein ligand ("peptide agonists") or, through competitive binding, inhibit the bioactivity of the large protein ligand ("peptide antagonists"). Phage display peptide libraries have emerged as a powerful method in identifying such peptide agonists and antagonists. In such libraries, random peptide sequences are displayed by fusion with coat proteins of filamentous phage. Typically,
25 the displayed peptides are affinity-eluted against an immobilized extracellular domain of an antigen or receptor. The retained phages may be enriched by successive rounds of affinity purification and repropagation. The best binding peptides may be sequenced to identify key residues within one or more structurally related families of peptides. The peptide sequences may also suggest which residues may be safely replaced by
30 alanine scanning or by mutagenesis at the DNA level. Mutagenesis libraries may be created and screened to further optimize the sequence of the best binders. *See, e.g.*, WO 0024782, published May 4, 2000, and the references cited therein; U.S. Patent No.

6,090,382 to Salfeld et al., issued July 18, 2000; WO 93/06213, to Hoogenboom et al., published Apr. 1, 1993.

Other display library screening method are known as well. For example, *E. coli* displays employ a peptide library fused to either the carboxyl terminus of the lac-repressor or the peptidoglycan-associated lipoprotein, and expressed in *E. coli*. Ribosome display involves halting the translation of random RNAs prior to ribosome release, resulting in a library of polypeptides with their associated RNAs still attached. RNA-peptide screening employs chemical linkage of peptides to RNA. Additionally, chemically derived peptide libraries have been developed in which peptides are immobilized on stable, non-biological materials, such as polyethylene rods or solvent-permeable resins. Another chemically derived peptide library uses photolithography to scan peptides immobilized on glass slides. These methods of chemical-peptide screening may be advantageous because they allow use of D-amino acids and other unnatural analogues, as well as non-peptide elements. See WO 0024782, published May 4, 2000, and the references cited therein.

Moreover, structural analysis of protein-protein interaction may also be used to suggest peptides that mimic the binding activity of large protein ligands. In such an analysis, the crystal structure may suggest the identity and relative orientation of critical residues of the large protein ligand, from which a peptide may be designed. These analytical methods may also be used to investigate the interaction between a receptor protein and peptides selected by phage display, which may suggest further modification of the peptides to increase binding affinity. Thus, conceptually, one may discover peptide mimetics of any protein using phage display and the other methods mentioned above. For example, these methods provide for epitope mapping, for identification of critical amino acids in protein-protein interactions, and as leads for the discovery of new therapeutic agents. See WO 0024782, published May 4, 2000, and the references cited therein.

Additionally, target-binding moieties produced synthetically are another alternative or additional moiety that may be included in the pseudo-antibody constructs of the present invention. For example, solution-phase synthesis has been used to create the eptifibatide molecule that binds the platelet receptor glycoprotein IIb/IIIa of human platelets, thus inhibiting platelet aggregation. Eptifibatide, sold commercially as

INTEGRILIN® (COR Therapeutics, Belmont, Cal.), is a cyclic heptapeptide containing six amino acids and one mercaptopropionyl (des-amino cycteiny) residue. An interdisulfide bridge is formed between the cysteine amide and the mercaptopropionyl moieties. This synthetic peptide is bound to X as shown in

5 Example 9, below, wherein X is or contains a functional group capable of forming the pseudo-antibody structure. The position of X is selected at any of those sites on the molecule at which substitution will retain some activity of the parent structure. In this specific example, the X may be a thiol group attached directly to the proline ring, or attached by way of an alkyl chain. X may also be carboxylic acid attached to the

10 proline ring, or attached by way of an alkyl chain or any other functional group that would allow it to be attached covalently to the branching moiety that serves to construct the pseudo-antibody.

The nature and source of the target-binding moiety of the pseudo-antibody of the present invention is not limited. The following is a general discussion of the variety

15 of proteins, peptides and biological molecules that may be used in the in accordance with the teachings herein. These descriptions do not serve to limit the scope of the invention, but rather illustrate the breadth of the invention.

Thus, an embodiment of the present invention may target one or more growth factors, or, conversely, derive the target-binding moiety from one or more growth

20 factors. Briefly, growth factors are hormones or cytokine proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation. Many growth factors are quite versatile, stimulating cellular division in numerous different cell types; while others are specific to a particular cell-type. The following Table 1 presents several factors, but is not intended to be

25 comprehensive or complete, yet introduces some of the more commonly known factors and their principal activities.

Table 1: Growth Factors

Factor	Principal Source	Primary Activity	Comments
Platelet Derived Growth Factor (PDGF)	Platelets, endothelial cells, placenta.	Promotes proliferation of connective tissue, glial and smooth muscle cells. PDGF receptor has intrinsic tyrosine kinase activity.	Dimer required for receptor binding. Two different protein chains, A and B, form 3 distinct dimer forms.
Epidermal Growth Factor (EGF)	Submaxillary gland, Brunners gland.	promotes proliferation of mesenchymal, glial and epithelial cells	EGF receptor has tyrosine kinase activity, activated in

Factor	Principal Source	Primary Activity	Comments
			response to EGF binding.
Fibroblast Growth Factor (FGF)	Wide range of cells; protein is associated with the ECM; nineteen family members. Receptors widely distributed in bone, implicated in several bone-related diseases.	Promotes proliferation of many cells including skeletal and nervous system; inhibits some stem cells; induces mesodermal differentiation. Non-proliferative effects include regulation of pituitary and ovarian cell function.	Four distinct receptors, all with tyrosine kinase activity. FGF implicated in mouse mammary tumors and Kaposi's sarcoma.
NGF		Promotes neurite outgrowth and neural cell survival	Several related proteins first identified as proto-oncogenes; <i>trkA</i> (<i>trackA</i>), <i>trkB</i> , <i>trkC</i>
Erythropoietin (Epo)	Kidney	Promotes proliferation and differentiation of erythrocytes	Also considered a 'blood protein,' and a colony stimulating factor.
Transforming Growth Factor α (TGF- α)	Common in transformed cells, found in macrophages and keratinocytes	Potent keratinocyte growth factor.	Related to EGF.
Transforming Growth Factor β (TGF- β)	Tumor cells, activated TH ₁ cells (T-helper) and natural killer (NK) cells	Anti-inflammatory (suppresses cytokine production and class II MHC expression), proliferative effects on many mesenchymal and epithelial cell types, may inhibit macrophage and lymphocyte proliferation.	Large family of proteins including activin, inhibin and bone morpho-genetic protein. Several classes and subclasses of cell-surface receptors
Insulin-Like Growth Factor-I (IGF-I)	Primarily liver, produced in response to GH and then induces subsequent cellular activities, particularly on bone growth	Promotes proliferation of many cell types, autocrine and paracrine activities in addition to the initially observed endocrine activities on bone.	Related to IGF-II and proinsulin, also called Somatomedin C. IGF-I receptor, like the insulin receptor, has intrinsic tyrosine kinase activity. IGF-I can bind to the insulin receptor.
Insulin-Like Growth Factor-II (IGF-II)	Expressed almost exclusively in embryonic and neonatal tissues.	Promotes proliferation of many cell types primarily of fetal origin. Related to IGF-I and proinsulin.	IGF-II receptor is identical to the mannose-6-phosphate receptor that is responsible for the integration of lysosomal enzymes

Additional growth factors that may be produced in accordance with the present invention include Activin (Vale et al., 321 NATURE 776 (1986); Ling et al., 321

NATURE 779 (1986)), Inhibin (U.S. Patent Nos. 4,737,578; 4,740,587), and Bone Morphogenic Proteins (BMPs) (U.S. Patent No. 5,846,931; Wozney, CELLULAR & MOLECULAR BIOLOGY OF BONE 131-167 (1993).

In addition to the growth factors discussed above, the present invention may target or use other cytokines. Secreted primarily from leukocytes, cytokines stimulate both the humoral and cellular immune responses, as well as the activation of phagocytic cells. Cytokines that are secreted from lymphocytes are termed lymphokines, whereas those secreted by monocytes or macrophages are termed monokines. A large family of cytokines are produced by various cells of the body. Many of the lymphokines are also known as interleukins (ILs), because they are not only secreted by leukocytes, but are also able to affect the cellular responses of leukocytes. More specifically, interleukins are growth factors targeted to cells of hematopoietic origin. The list of identified interleukins grows continuously. *See, e.g.*, U.S. Patent No. 6,174,995; U.S. Patent No. 6,143,289; Sallusto et al., 18 ANNU. REV. IMMUNOL. 593 (2000) Kunkel et al., 59 J. LEUKOCYTE BIOL. 81 (1996).

Additional growth factor/cytokines encompassed in the present invention include pituitary hormones such as human growth hormone (HGH), follicle stimulating hormones (FSH, FSH α , and FSH β), Human Chorionic Gonadotrophins (HCG, HCG α , HCG β), uFSH (urofollitropin), Gonatropin releasing hormone (GRH), Growth Hormone (GH), leuteinizing hormones (LH, LH α , LH β), somatostatin, prolactin, thyrotropin (TSH, TSH α , TSH β), thyrotropin releasing hormone (TRH), parathyroid hormones, estrogens, progesterones, testosterone, or structural or functional analog thereof. All of these proteins and peptides are known in the art.

The cytokine family also includes tumor necrosis factors, colony stimulating factors, and interferons. *See, e.g.*, Cosman, 7 BLOOD CELL (1996); Gruss et al., 85 BLOOD 3378 (1995); Beutler et al., 7 ANNU. REV. IMMUNOL. 625 (1989); Aggarwal et al., 260 J. BIOL. CHEM. 2345 (1985); Pennica et al., 312 NATURE 724 (1984); R & D Systems, CYTOKINE MINI-REVIEWS, at <http://www.rndsystems.com>.

Several cytokines are introduced, briefly, in Table 2 below.

Table 2: Cytokines

Cytokine	Principal Source	Primary Activity
Interleukins IL1-a and -b	Primarily macrophages but also neutrophils, endothelial cells, smooth muscle cells, glial cells, astrocytes, B- and T-cells, fibroblasts, and	Costimulation of APCs and T cells; stimulates IL-2 receptor production and expression of interferon- γ , may induce proliferation in non-lymphoid cells.

Cytokine	Principal Source	Primary Activity
	keratinocytes.	
IL-2	CD4+ T-helper cells, activated TH ₁ cells, NK cells.	Major interleukin responsible for clonal T-cell proliferation. IL-2 also exerts effects on B-cells, macrophages, and natural killer (NK) cells. IL-2 receptor is not expressed on the surface of resting T-cells, but expressed constitutively on NK cells, that will secrete TNF- α , IFN- γ and GM-CSF in response to IL-2, which in turn activate macrophages.
IL-3	Primarily T-cells	Also known as multi-CSF, as it stimulates stem cells to produce all forms of hematopoietic cells.
IL-4	TH ₂ and mast cells	B cell proliferation, eosinophil and mast cell growth and function, IgE and class II MHC expression on B cells, inhibition of monokine production
IL-5	TH ₂ and mast cells	eosinophil growth and function
IL-6	Macrophages, fibroblasts, endothelial cells and activated T-helper cells. Does not induce cytokine expression.	IL-6 acts in synergy with IL-1 and TNF- α in many immune responses, including T-cell activation; primary inducer of the acute-phase response in liver; enhances the differentiation of B-cells and their consequent production of immunoglobulin; enhances Glucocorticoid synthesis.
IL-7	thymic and marrow stromal cells	T and B lymphopoiesis
IL-8	Monocytes, neutrophils, macrophages, and NK cells.	Chemoattractant (chemokine) for neutrophils, basophils and T-cells; activates neutrophils to degranulate.
IL-9	T cells	hematopoietic and thymopoietic effects
IL-10	activated TH ₂ cells, CD8 ⁺ T and B cells, macrophages	inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, mast cell growth
IL-11	stromal cells	synergistic hematopoietic and thrombopoietic effects
IL-12	B cells, macrophages	proliferation of NK cells, INF- γ production, promotes cell-mediated immune functions
IL-13	TH ₂ cells	IL-4-like activities
IL-18	macrophages/Kupffer cells, keratinocytes, glucocorticoid-secreting adrenal cortex cells, and osteoblasts	Interferon-gamma-inducing factor with potent pro-inflammatory activity
IL-21	Activated T cells	IL21 has a role in proliferation and maturation of natural killer (NK) cell populations from bone marrow, in the proliferation of mature B-cell populations co-stimulated with anti-CD40, and in the proliferation of T cells co-stimulated with anti-CD3.

Cytokine	Principal Source	Primary Activity
IL-23	Activated dendritic cells	A complex of p19 and the p40 subunit of IL-12. IL-23 binds to IL-12R beta 1 but not IL-12R beta 2; activates Stat4 in PHA blast T cells; induces strong proliferation of mouse memory T cells; stimulates IFN-gamma production and proliferation in PHA blast T cells, as well as in CD45RO (memory) T cells.
TumorNecrosis Factor TNF- α	Primarily activated macrophages.	Once called cachectin; induces the expression of other autocrine growth factors, increases cellular responsiveness to growth factors; induces signaling pathways that lead to proliferation; induces expression of a number of nuclear proto-oncogenes as well as of several interleukins.
(TNF- β)	T-lymphocytes, particularly cytotoxic T-lymphocytes (CTL cells); induced by IL-2 and antigen-T-Cell receptor interactions.	Also called lymphotoxin; kills a number of different cell types, induces terminal differentiation in others; inhibits lipoprotein lipase present on the surface of vascular endothelial cells.
Interferons INF- α and - β	macrophages, neutrophils and some somatic cells	Known as type I interferons; antiviral effect; induction of class I MHC on all somatic cells; activation of NK cells and macrophages.
Interferon INF- γ	Primarily CD8+ T-cells, activated TH ₁ and NK cells	Type II interferon; induces of class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, activates macrophages, neutrophils, NK cells, promotes cell-mediated immunity, enhances ability of cells to present antigens to T-cells; antiviral effects.
Monocyte Chemoattractant Protein-1 (MCP1)	Peripheral blood monocytes/macrophages	Attracts monocytes to sites of vascular endothelial cell injury, implicated in atherosclerosis.
Colony Stimulating Factors (CSFs)		Stimulate the proliferation of specific pluripotent stem cells of the bone marrow in adults.
Granulocyte-CSF (G-CSF)		Specific for proliferative effects on cells of the granulocyte lineage; proliferative effects on both classes of lymphoid cells.
Macrophage-CSF (M-CSF)		Specific for cells of the macrophage lineage.
Granulocyte-MacrophageCSF (GM-CSF)		Proliferative effects on cells of both the macrophage and granulocyte lineages.

Other cytokines of interest that may be produced by the invention described herein include adhesion molecules(R. & D Systems, ADHESION MOLECULES I (1996),

available at <http://www.rndsystems.com>); angiogenin (U.S. Patent No. 4,721,672; Moener et al., 226 EUR. J. BIOCHEM. 483 (1994)); annexin V (Cookson et al., 20 GENOMICS 463 (1994); Grundmann et al., 85 PROC. NATL. ACAD. SCI. USA 3708 (1988); U.S. Patent No. 5,767,247); caspases (U.S. Patent No. 6,214,858; Thornberry et al., 281 SCIENCE 1312 (1998)); chemokines (U.S. Patent Nos. 6,174,995; 6,143,289; Sallusto et al., 18 ANNU. REV. IMMUNOL. 593 (2000) Kunkel et al., 59 J. LEUKOCYTE BIOL. 81 (1996)); endothelin (U.S. Patent Nos. 6,242,485; 5,294,569; 5,231,166); eotaxin (U.S. Patent No. 6,271,347; Ponath et al., 97(3) J. CLIN. INVEST. 604-612 (1996)); Flt-3 (U.S. Patent No. 6,190,655); heregulins (U.S. Patent Nos. 6,284,535; 6,143,740; 6,136,558; 5,859,206; 5,840,525); Leptin (Leroy et al., 271(5) J. BIOL. CHEM. 2365 (1996); Maffei et al., 92 PNAS 6957 (1995); Zhang Y. et al. (1994) NATURE 372: 425-432); Macrophage Stimulating Protein (MSP) (U.S. Patent Nos. 6,248,560; 6,030,949; 5,315,000); Neurotrophic Factors (U.S. Patent Nos. 6,005,081; 5,288,622); Pleiotrophin/Midkine (PTN/MK) (Pedraza et al., 117 J. BIOCHEM. 845 (1995); Tamura et al., 3 ENDOCRINE 21 (1995); U.S. Patent No. 5,210,026; Kadomatsu et al., 151 BIOCHEM. BIOPHYS. RES. COMMUN. 1312 (1988)); STAT proteins (U.S. Patent Nos. 6,030,808; 6,030,780; Darnell et al., 277 SCIENCE 1630-1635 (1997)); Tumor Necrosis Factor Family (Cosman, 7 BLOOD CELL (1996); Gruss et al., 85 BLOOD 3378 (1995); Beutler et al., 7 ANNU. REV. IMMUNOL. 625 (1989); Aggarwal et al., 260 J. BIOL. CHEM. 2345 (1985); Pennica et al., 312 NATURE 724 (1984).

Also of interest regarding cytokines are proteins or chemical moieties that interact with cytokines, such as Matrix Metalloproteinases (MMPs) (U.S. Patent No. 6,307,089; NAGASE, MATRIX METALLOPROTEINASES IN ZINC METALLOPROTEASES IN HEALTH AND DISEASE (1996)), and Nitric Oxide Synthases (NOS) (Fukuto, 34 ADV. PHARM 1 (1995); U.S. Patent No. 5,268,465).

The present invention may also be used to affect blood proteins, a generic name for a vast group of proteins generally circulating in blood plasma, and important for regulating coagulation and clot dissolution. *See, e.g.*, Haematologic Technologies, Inc., HTI CATALOG, available at www.haemtech.com. Table 3 introduces, in a non-limiting fashion, some of the blood proteins contemplated by the present invention.

Table 3: Blood Proteins

Protein	Principle Activity	Reference
Factor V	In coagulation, this glycoprotein pro-cofactor, is converted to active cofactor,	Mann et al., 57 ANN. REV. BIOCHEM. 915 (1988); <i>see also</i> Nesheim et al., 254

Protein	Principle Activity	Reference
	factor Va, via the serine protease α -thrombin, and less efficiently by its serine protease cofactor Xa. The prothrombinase complex rapidly converts zymogen prothrombin to the active serine protease, α -thrombin. Down regulation of prothrombinase complex occurs via inactivation of Va by activated protein C.	J. BIOL. CHEM. 508 (1979); Tracy et al., 60 BLOOD 59 (1982); Nesheim et al., 80 METHODS ENZYMOL. 249 (1981); Jenny et al., 84 PROC. NATL. ACAD. SCI. USA 4846 (1987).
Factor VII	Single chain glycoprotein zymogen in its native form. Proteolytic activation yields enzyme factor VIIa, which binds to integral membrane protein tissue factor, forming an enzyme complex that proteolytically converts factor X to Xa. Also known as extrinsic factor Xase complex. Conversion of VII to VIIa catalyzed by a number of proteases including thrombin, factors IXa, Xa, XIa, and XIIa. Rapid activation also occurs when VII combines with tissue factor in the presence of Ca, likely initiated by a small amount of pre-existing VIIa. Not readily inhibited by antithrombin III/heparin alone, but is inhibited when tissue factor added.	<i>See generally</i> , Broze et al., 80 METHODS ENZYMOL. 228 (1981); Bajaj et al., 256 J. BIOL. CHEM. 253 (1981); Williams et al., 264 J. BIOL. CHEM. 7536 (1989); Kisiel et al., 22 THROMBOSIS RES. 375 (1981); Seligsohn et al., 64 J. CLIN. INVEST. 1056 (1979); Lawson et al., 268 J. BIOL. CHEM. 767 (1993).
Factor IX	Zymogen factor IX, a single chain vitamin K-dependent glycoprotein, made in liver. Binds to negatively charged phospholipid surfaces. Activated by factor XIa or the factor VIIa/tissue factor/phospholipid complex. Cleavage at one site yields the intermediate IX α , subsequently converted to fully active form IXa β by cleavage at another site. Factor IXa β is the catalytic component of the "intrinsic factor Xase complex" (factor VIIIa/IXa/Ca ²⁺ /phospholipid) that proteolytically activates factor X to factor Xa.	Thompson, 67 BLOOD, 565 (1986); Hedner et al., HEMOSTASIS AND THROMBOSIS 39-47 (R.W. Colman, J. Hirsh, V.J. Marder, E.W. Salzman ed., 2 nd ed. J.P. Lippincott Co., Philadelphia) 1987; Fujikawa et al., 45 METHODS IN ENZYMOLOGY 74 (1974).
Factor X	Vitamin K-dependent protein zymogen, made in liver, circulates in plasma as a two chain molecule linked by a disulfide bond. Factor Xa (activated X) serves as the enzyme component of prothrombinase complex, responsible for rapid conversion of prothrombin to thrombin.	<i>See</i> Davie et al., 48 ADV. ENZYMOL. 277 (1979); Jackson, 49 ANN. REV. BIOCHEM. 765 (1980); <i>see also</i> Fujikawa et al., 11 BIOCHEM. 4882 (1972); Discipio et al., 16 BIOCHEM. 698 (1977); Discipio et al., 18 BIOCHEM. 899 (1979); Jackson et al., 7 BIOCHEM. 4506 (1968); McMullen et al., 22 BIOCHEM. 2875 (1983).
Factor XI	Liver-made glycoprotein homodimer circulates, in a non-covalent complex	Thompson et al., 60 J. CLIN. INVEST. 1376 (1977); Kurachi et al., 16

Protein	Principle Activity	Reference
	with high molecular weight kininogen, as a zymogen, requiring proteolytic activation to acquire serine protease activity. Conversion of factor XI to factor XIa is catalyzed by factor XIIa. XIa unique among the serine proteases, since it contains two active sites per molecule. Works in the intrinsic coagulation pathway by catalyzing conversion of factor IX to factor IXa. Complex form, factor XIa/HMWK, activates factor XII to factor XIIa and prekallikrein to kallikrein. Major inhibitor of XIa is α_1 -antitrypsin and to lesser extent, antithrombin-III. Lack of factor XI procoagulant activity causes bleeding disorder: plasma thromboplastin antecedent deficiency.	BIOCHEM. 5831 (1977); Bouma et al., 252 J. BIOL. CHEM. 6432 (1977); Wuepper, 31 FED. PROC. 624 (1972); Saito et al., 50 BLOOD 377 (1977); Fujikawa et al., 25 BIOCHEM. 2417 (1986); Kurachi et al., 19 BIOCHEM. 1330 (1980); Scott et al., 69 J. CLIN. INVEST. 844 (1982).
Factor XII (Hageman Factor)	Glycoprotein zymogen. Reciprocal activation of XII to active serine protease factor XIIa by kallikrein is central to start of intrinsic coagulation pathway. Surface bound α -XIIa activates factor XI to XIa. Secondary cleavage of α -XIIa by kallikrein yields β -XIIa, and catalyzes solution phase activation of kallikrein, factor VII and the classical complement cascade.	Schmaier et al., 18-38, and Davie, 242-267 HEMOSTASIS & THROMBOSIS (Colman et al., eds., J.B. Lippincott Co., Philadelphia, 1987).
Factor XIII	Zymogenic form of glutamyl-peptide γ -glutamyl transferase factor XIIIa (fibrinolygase, plasma transglutaminase, fibrin stabilizing factor). Made in the liver, found extracellularly in plasma and intracellularly in platelets, megakaryocytes, monocytes, placenta, uterus, liver and prostrate tissues. Circulates as a tetramer of 2 pairs of nonidentical subunits (A_2B_2). Full expression of activity is achieved only after the Ca^{2+} - and fibrin(ogen)-dependent dissociation of B subunit dimer from A_2 ' dimer. Last of the zymogens to become activated in the coagulation cascade, the only enzyme in this system that is not a serine protease. XIIIa stabilizes the fibrin clot by crosslinking the α and γ -chains of fibrin. Serves in cell proliferation in wound healing, tissue remodeling, atherosclerosis, and tumor growth.	See McDonough, 340-357 HEMOSTASIS & THROMBOSIS (Colman et al., eds., J.B. Lippincott Co., Philadelphia, 1987); Folk et al., 113 METHODS ENZYMOL. 364 (1985); Greenberg et al., 69 BLOOD 867 (1987). Other proteins known to be substrates for Factor XIIIa, that may be hemostatically important, include fibronectin (Iwanaga et al., 312 ANN. NY ACAD. SCI. 56 (1978)), a_2 -antiplasmin (Sakata et al., 65 J. CLIN. INVEST. 290 (1980)), collagen (Mosher et al., 64 J. CLIN. INVEST. 781 (1979)), factor V (Francis et al., 261 J. BIOL. CHEM. 9787 (1986)), von Willebrand Factor (Mosher et al., 64 J. CLIN. INVEST. 781 (1979)) and thrombospondin (Bale et al., 260 J. BIOL. CHEM. 7502 (1985); Bohn, 20 MOL. CELL BIOCHEM. 67 (1978)).
Fibrinogen	Plasma fibrinogen, a large glycoprotein, disulfide linked dimer made of 3 pairs of	FURLAN, <i>Fibrinogen</i> , IN HUMAN PROTEIN DATA, (Haeberli, ed., VCH

Protein	Principle Activity	Reference
	<p>non-identical chains (Aa, Bb and g), made in liver. Aa has N-terminal peptide (fibrinopeptide A (FPA), factor XIIIa crosslinking sites, and 2 phosphorylation sites. Bb has fibrinopeptide B (FPB), 1 of 3 N-linked carbohydrate moieties, and an N-terminal pyroglutamic acid. The g chain contains the other N-linked glycos. site, and factor XIIIa cross-linking sites. Two elongated subunits ((AaBbg)₂) align in an antiparallel way forming a trinodular arrangement of the 6 chains. Nodes formed by disulfide rings between the 3 parallel chains. Central node (n-disulfide knot, E domain) formed by N-termini of all 6 chains held together by 11 disulfide bonds, contains the 2 IIa-sensitive sites. Release of FPA by cleavage generates Fbn I, exposing a polymerization site on Aa chain. These sites bind to regions on the D domain of Fbn to form proto-fibrils. Subsequent IIa cleavage of FPB from the Bb chain exposes additional polymerization sites, promoting lateral growth of Fbn network. Each of the 2 domains between the central node and the C-terminal nodes (domains D and E) has parallel α-helical regions of the Aa, Bb and g chains having protease-(plasmin-) sensitive sites. Another major plasmin sensitive site is in hydrophilic preturbance of α-chain from C-terminal node. Controlled plasmin degradation converts Fbg into fragments D and E.</p>	<p>Publishers, N.Y., 1995); Doolittle, in HAEMOSTASIS & THROMBOSIS, 491-513 (3rd ed., Bloom et al., eds., Churchill Livingstone, 1994); HANTGAN, et al., in HAEMOSTASIS & THROMBOSIS 269-89 (2d ed., Forbes et al., eds., Churchill Livingstone, 1991).</p>
Fibronectin	<p>High molecular weight, adhesive, glycoprotein found in plasma and extracellular matrix in slightly different forms. Two peptide chains interconnected by 2 disulfide bonds, has 3 different types of repeating homologous sequence units. Mediates cell attachment by interacting with cell surface receptors and extracellular matrix components. Contains an Arg-Gly-Asp-Ser (RGDS) cell attachment-promoting sequence, recognized by specific cell receptors, such as those on platelets. Fibrin-fibronectin complexes stabilized by factor XIIIa-catalyzed covalent cross-linking of fibronectin to the fibrin a chain.</p>	<p>Skorstengaard et al., 161 Eur. J. BIOCHEM. 441 (1986); Kornblihtt et al., 4 EMBO J. 1755 (1985); Odermatt et al., 82 PNAS 6571 (1985); Hynes, R.O., ANN. REV. CELL BIOL., 1, 67 (1985); Mosher 35 ANN. REV. MED. 561 (1984); Rouslahti et al., 44 Cell 517 (1986); Hynes 48 CELL 549 (1987); Mosher 250 BIOL. CHEM. 6614 (1975).</p>

Protein	Principle Activity	Reference
b ₂ - Glycoprotein I	Also called b ₂ I and Apolipoprotein H. Highly glycosylated single chain protein made in liver. Five repeating mutually homologous domains consisting of approximately 60 amino acids disulfide bonded to form Short Consensus Repeats (SCR) or Sushi domains. Associated with lipoproteins, binds anionic surfaces like anionic vesicles, platelets, DNA, mitochondria, and heparin. Binding can inhibit contact activation pathway in blood coagulation. Binding to activated platelets inhibits platelet associated prothrombinase and adenylate cyclase activities. Complexes between b ₂ I and cardiolipin have been implicated in the anti-phospholipid related immune disorders LAC and SLE.	<i>See, e.g.</i> , Lozier et al., 81 PNAS 2640-44 (1984); Kato & Enjyoi 30 BIOCHEM. 11687-94 (1997); Wurm, 16 INT'L J. BIOCHEM. 511-15 (1984); Bendixen et al., 31 BIOCHEM. 3611-17 (1992); Steinkasserer et al., 277 BIOCHEM. J. 387-91 (1991); Nimpf et al., 884 BIOCHEM. BIOPHYS. ACTA 142-49 (1986); Kroll et al. 434 BIOCHEM. BIOPHYS. Acta 490-501 (1986); Polz et al., 11 INT'L J. BIOCHEM. 265-73 (1976); McNeil et al., 87 PNAS 4120-24 (1990); Galli et al., I LANCET 1544-47 (1990); Matsuuna et al., II LANCET 177-78 (1990); Pengo et al., 73 THROMBOSIS & HAEMOSTASIS 29-34 (1995).
Osteonectin	Acidic, noncollagenous glycoprotein (Mr=29,000) originally isolated from fetal and adult bovine bone matrix. May regulate bone metabolism by binding hydroxyapatite to collagen. Identical to human placental SPARC. An alpha granule component of human platelets secreted during activation. A small portion of secreted osteonectin expressed on the platelet cell surface in an activation-dependent manner	Villarreal et al., 28 BIOCHEM. 6483 (1989); Tracy et al., 29 INT'L J. BIOCHEM. 653 (1988); Romberg et al., 25 BIOCHEM. 1176 (1986); Sage & Bornstein 266 J. BIOL. CHEM. 14831 (1991); Kelm & Mann 4 J. BONE MIN. RES. 5245 (1989); Kelm et al., 80 BLOOD 3112 (1992).
Plasminogen	Single chain glycoprotein zymogen with 24 disulfide bridges, no free sulfhydryls, and 5 regions of internal sequence homology, "kringles", each five triple-looped, three disulfide bridged, and homologous to kringle domains in t-PA, u-PA and prothrombin. Interaction of plasminogen with fibrin and α 2-antiplasmin is mediated by lysine binding sites. Conversion of plasminogen to plasmin occurs by variety of mechanisms, including urinary type and tissue type plasminogen activators, streptokinase, staphylokinase, kallikrein, factors IXa and XIIa, but all result in hydrolysis at Arg560-Val561, yielding two chains that remain covalently associated by a disulfide bond.	<i>See</i> Robbins, 45 METHODS IN ENZYMOLOGY 257 (1976); COLLEN, 243-258 BLOOD COAG. (Zwaal et al., eds., New York, Elsevier, 1986); <i>see also</i> Castellino et al., 80 METHODS IN ENZYMOLOGY 365 (1981); Wohl et al., 27 THROMB. RES. 523 (1982); Barlow et al., 23 BIOCHEM. 2384 (1984); SOTTRUP-JENSEN ET AL., 3 PROGRESS IN CHEM. FIBRINOLYSIS & THROMBOLYSIS 197-228 (Davidson et al., eds., Raven Press, New York 1975).
tissue Plasminogen Activator	t-PA, a serine endopeptidase synthesized by endothelial cells, is the major physiologic activator of plasminogen in clots, catalyzing conversion of	<i>See</i> Plasminogen.

Protein	Principle Activity	Reference
	plasminogen to plasmin by hydrolising a specific arginine-alanine bond. Requires fibrin for this activity, unlike the kidney-produced version, urokinase-PA.	
Plasmin	<i>See Plasminogen.</i> Plasmin, a serine protease, cleaves fibrin, and activates and/or degrades compounds of coagulation, kinin generation, and complement systems. Inhibited by a number of plasma protease inhibitors <i>in vitro</i> . Regulation of plasmin <i>in vivo</i> occurs mainly through interaction with α_2 -antiplasmin, and to a lesser extent, α_2 -macroglobulin.	<i>See Plasminogen.</i>
Platelet Factor-4	Low molecular weight, heparin-binding protein secreted from agonist-activated platelets as a homotetramer in complex with a high molecular weight, proteoglycan, carrier protein. Lysine-rich, COOH-terminal region interacts with cell surface expressed heparin-like glycosaminoglycans on endothelial cells. PF-4 neutralizes anticoagulant activity of heparin exerts procoagulant effect, and stimulates release of histamine from basophils. Chemotactic activity toward neutrophils and monocytes. Binding sites on the platelet surface have been identified and may be important for platelet aggregation.	Rucinski et al., 53 BLOOD 47 (1979); Kaplan et al., 53 BLOOD 604 (1979); George 76 BLOOD 859 (1990); Busch et al., 19 THROMB. RES. 129 (1980); Rao et al., 61 BLOOD 1208 (1983); Brindley, et al., 72 J. CLIN. INVEST. 1218 (1983); Deuel et al., 74 PNAS 2256 (1981); Osterman et al., 107 BIOCHEM. BIOPHYS. RES. COMMUN. 130 (1982); Capitanio et al., 839 BIOCHEM. BIOPHYS. ACTA 161 (1985).
Protein C	Vitamin K-dependent zymogen, protein C, made in liver as a single chain polypeptide then converted to a disulfide linked heterodimer. Cleaving the heavy chain of human protein C converts the zymogen into the serine protease, activated protein C. Cleavage catalyzed by a complex of α -thrombin and thrombomodulin. Unlike other vitamin K dependent coagulation factors, activated protein C is an anticoagulant that catalyzes the proteolytic inactivation of factors Va and VIIIa, and contributes to the fibrinolytic response by complex formation with plasminogen activator inhibitors.	<i>See Esmon</i> , 10 PROGRESS IN THROMB. & HEMOSTAS. 25 (1984); Stenflo, 10 SEMIN. IN THROMB. & HEMOSTAS. 109 (1984); Griffen et al., 60 BLOOD 261 (1982); Kiesel et al., 80 METHODS ENZYMOL. 320 (1981); Discipio et al., 18 BIOCHEM. 899 (1979).
Protein S	Single chain vitamin K-dependent protein functions in coagulation and complement cascades. Does not possess the catalytic triad. Complexes to C4b binding protein (C4BP) and to negatively charged phospholipids,	Walker 10 SEMIN. THROMB. HEMOSTAS. 131 (1984); Dahlback et al., 10 SEMIN. THROMB. HEMOSTAS., 139 (1984); Walker 261 J. BIOL. CHEM. 10941 (1986).

Protein	Principle Activity	Reference
	concentrating C4BP at cell surfaces following injury. Unbound S serves as anticoagulant cofactor protein with activated Protein C. A single cleavage by thrombin abolishes protein S cofactor activity by removing gla domain.	
Protein Z	Vitamin K-dependent, single-chain protein made in the liver. Direct requirement for the binding of thrombin to endothelial phospholipids. Domain structure similar to that of other vitamin K-dependant zymogens like factors VII, IX, X, and protein C. N-terminal region contains carboxyglutamic acid domain enabling phospholipid membrane binding. C-terminal region lacks "typical" serine protease activation site. Cofactor for inhibition of coagulation factor Xa by serpin called protein Z-dependant protease inhibitor. Patients diagnosed with protein Z deficiency have abnormal bleeding diathesis during and after surgical events.	Sejima et al., 171 BIOCHEM. BIOPHYSICS RES. COMM. 661 (1990); Hogg et al., 266 J. BIOL. CHEM. 10953 (1991); Hogg et al., 17 BIOCHEM. BIOPHYSICS RES. COMM. 801 (1991); Han et al., 38 BIOCHEM. 11073 (1999); Kemkes-Matthes et al., 79 THROMB. RES. 49 (1995).
Prothrombin	Vitamin K-dependent, single-chain protein made in the liver. Binds to negatively charged phospholipid membranes. Contains two "kringle" structures. Mature protein circulates in plasma as a zymogen and, during coagulation, is proteolytically activated to the potent serine protease α -thrombin.	Mann et al., 45 METHODS IN ENZYMOLOGY 156 (1976); Magnusson et al., PROTEASES IN BIOLOGICAL CONTROL 123-149 (Reich et al., eds. Cold Spring Harbor Labs., New York 1975); Discipio et al., 18 BIOCHEM. 899 (1979).
α -Thrombin	See Prothrombin. During coagulation, thrombin cleaves fibrinogen to form fibrin, the terminal proteolytic step in coagulation, forming the fibrin clot. Thrombin also responsible for feedback activation of procofactors V and VIII. Activates factor XIII and platelets, functions as vasoconstrictor protein. Procoagulant activity arrested by heparin cofactor II or the antithrombin III/heparin complex, or complex formation with thrombomodulin. Formation of thrombin/thrombomodulin complex results in inability of thrombin to cleave fibrinogen and activate factors V and VIII, but increases the efficiency of thrombin for activation of the anticoagulant, protein C.	45 METHODS ENZYMOL. 156 (1976).
b-Thromboglobulin	Low molecular weight, heparin-binding, platelet-derived tetramer protein, consisting of four identical peptide	See, e.g., George 76 BLOOD 859 (1990); Holt & Niewiarowski 632 BIOCHIM. BIOPHYS. ACTA 284 (1980);

Protein	Principle Activity	Reference
	chains. Lower affinity for heparin than PF-4. Chemotactic activity for human fibroblasts, other functions unknown.	Niewiarowski et al., 55 BLOOD 453 (1980); Varma et al., 701 BIOCHIM. BIOPHYS. ACTA 7 (1982); Senior et al., 96 J. CELL. BIOL. 382 (1983).
Thrombopoietin	Human TPO (Thrombopoietin, Mpl-ligand, MGDF) stimulates the proliferation and maturation of megakaryocytes and promotes increased circulating levels of platelets <i>in vivo</i> . Binds to c-Mpl receptor.	Horikawa et al., 90(10) BLOOD 4031-38 (1997); de Sauvage et al., 369 NATURE 533-58 (1995).
Thrombospondin	High-molecular weight, heparin-binding glycoprotein constituent of platelets, consisting of three, identical, disulfide-linked polypeptide chains. Binds to surface of resting and activated platelets, may effect platelet adherence and aggregation. An integral component of basement membrane in different tissues. Interacts with a variety of extracellular macromolecules including heparin, collagen, fibrinogen and fibronectin, plasminogen, plasminogen activator, and osteonectin. May modulate cell-matrix interactions.	Dawes et al., 29 THROMB. RES. 569 (1983); Switalska et al., 106 J. LAB. CLIN. MED. 690 (1985); Lawler et al. 260 J. BIOL. CHEM. 3762 (1985); Wolff et al., 261 J. BIOL. CHEM. 6840 (1986); Asch et al., 79 J. CLIN. CHEM. 1054 (1987); Jaffe et al., 295 NATURE 246 (1982); Wright et al., 33 J. HISTOCHEM. CYTOCHEM. 295 (1985); Dixit et al., 259 J. BIOL. CHEM. 10100 (1984); Mumby et al., 98 J. CELL. BIOL. 646 (1984); Lahav et al., 145 EUR. J. BIOCHEM. 151 (1984); Silverstein et al., 260 J. BIOL. CHEM. 10346 (1985); Clezardin et al. 175 EUR. J. BIOCHEM. 275 (1988); Sage & Bornstein (1991).
Von Willebrand Factor	Multimeric plasma glycoprotein made of identical subunits held together by disulfide bonds. During normal hemostasis, larger multimers of vWF cause platelet plug formation by forming a bridge between platelet glycoprotein IB and exposed collagen in the subendothelium. Also binds and transports factor VIII (antihemophilic factor) in plasma.	Hoyer 58 BLOOD 1 (1981); Ruggeri & Zimmerman 65 J. CLIN. INVEST. 1318 (1980); Hoyer & Shainoff 55 BLOOD 1056 (1980); Meyer et al., 95 J. LAB. CLIN. INVEST. 590 (1980); Santoro 21 THROMB. RES. 689 (1981); Santoro, & Cowan 2 COLLAGEN RELAT. RES. 31 (1982); Morton et al., 32 THROMB. RES. 545 (1983); Tuddenham et al., 52 BRIT. J. HAEMATOL. 259 (1982).

Additional blood proteins contemplated herein include the following human serum proteins, which may also be placed in another category of protein (such as hormone or antigen): Actin, Actinin, Amyloid Serum P, Apolipoprotein E, B2-

5 Microglobulin, C-Reactive Protein (CRP), Cholesterylester transfer protein (CETP), Complement C3B, Ceruplasmin, Creatine Kinase, Cystatin, Cytokeratin 8, Cytokeratin 14, Cytokeratin 18, Cytokeratin 19, Cytokeratin 20, Desmin, Desmocollin 3, FAS (CD95), Fatty Acid Binding Protein, Ferritin, Filamin, Glial Filament Acidic Protein, Glycogen Phosphorylase Isoenzyme BB (GPBB), Haptoglobulin, Human Myoglobin,

10 Myelin Basic Protein, Neurofilament, Placental Lactogen, Human SHBG, Human

Thyroid Peroxidase, Receptor Associated Protein, Human Cardiac Troponin C, Human Cardiac Troponin I, Human Cardiac Troponin T, Human Skeletal Troponin I, Human Skeletal Troponin T, Vimentin, Vinculin, Transferrin Receptor, Prealbumin, Albumin, Alpha-1-Acid Glycoprotein, Alpha-1-Antichymotrypsin, Alpha-1-Antitrypsin, Alpha-
5 Fetoprotein, Alpha-1-Microglobulin, Beta-2-microglobulin, C-Reactive Protein, Haptoglobin, Myoglobin, Prealbumin, PSA, Prostatic Acid Phosphatase, Retinol Binding Protein, Thyroglobulin, Thyroid Microsomal Antigen, Thyroxine Binding Globulin, Transferrin, Troponin I, Troponin T, Prostatic Acid Phosphatase, Retinol Binding Globulin (RBP). All of these proteins, and sources thereof, are known in the
10 art. Many of these proteins are available commercially from, for example, Research Diagnostics, Inc. (Flanders, N.J.).

The pseudo-antibody of the present invention may also incorporate or target neurotransmitters, or functional portions thereof. Neurotransmitters are chemicals made by neurons and used by them to transmit signals to the other neurons or non-
15 neuronal cells (e.g., skeletal muscle; myocardium, pineal glandular cells) that they innervate. Neurotransmitters produce their effects by being released into synapses when their neuron of origin fires (i.e., becomes depolarized) and then attaching to receptors in the membrane of the post-synaptic cells. This causes changes in the fluxes of particular ions across that membrane, making cells more likely to become
20 depolarized, if the neurotransmitter happens to be excitatory, or less likely if it is inhibitory. Neurotransmitters can also produce their effects by modulating the production of other signal-transducing molecules ("second messengers") in the post-synaptic cells. *See generally* COOPER, BLOOM & ROTH, THE BIOCHEMICAL BASIS OF NEUROPHARMACOLOGY (7th Ed. Oxford Univ. Press, NYC, 1996);
25 <http://web.indstate.edu/thcme/mwking/nerves>. Neurotransmitters contemplated in the present invention include, but are not limited to, Acetylcholine, Serotonin, γ -aminobutyrate (GABA), Glutamate, Aspartate, Glycine, Histamine, Epinephrine, Norepinephrine, Dopamine, Adenosine, ATP, Nitric oxide, and any of the peptide neurotransmitters such as those derived from pre-opiomelanocortin (POMC), as well as
30 antagonists and agonists of any of the foregoing.

Numerous other proteins or peptides may serve as either targets, or as a source of target-binding moieties as described herein. Table 4 presents a non-limiting list and description of some pharmacologically active peptides which may serve as, or serve as

a source of a functional derivative of, a portion of a pseudo-antibody of the present invention.

Table 4: Pharmacologically active peptides

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
EPO receptor (intrapeptide disulfide-bonded)	EPO mimetic	Wrighton et al., 273 SCIENCE 458-63 (1996); U.S. Pat. No. 5,773,569, issued June 30, 1998.
EPO receptor (C-terminally cross- linked dimer)	EPO mimetic	Livnah et al., 273 SCIENCE 464-71 (1996); Wrighton et al., 15 NATURE BIOTECHNOLOGY 1261-5 (1997); Int'l Patent Application WO 96/40772, published Dec. 19, 1996.
EPO receptor (linear)	EPO mimetic	Naranda et al., 96 PNAS 7569-74 (1999).
c-Mpl (linear)	TPO-mimetic	Cwirla et al., 276 SCIENCE 1696-9 (1997); U.S. Pat. No. 5,869,451, issued Feb. 9, 1999; U.S. Pat. No. 5,932,946, issued Aug. 3, 1999.
c-Mpl (C-terminally cross- linked dimer)	TPO-mimetic	Cwirla et al., 276 SCIENCE 1696-9 (1997).
(disulfide-linked dimer)	stimulation of hematopoiesis ("G-CSF-mimetic")	Paukovits et al., 364 HOPPE-SEYLER'S Z. PHYSIOL. CHEM. 30311 (1984); Laerungal., 16 EXP. HEMAT. 274-80 (1988).
(alkylene-linked dimer)	G-CSF-mimetic	Batnagar et al., 39 J. MED. CHEM. 38149 (1996); Cuthbertson et al., 40 J. MED. CHEM. 2876-82 (1997); King et al., 19 EXP. HEMATOL. 481 (1991); King et al., 86(Suppl. 1) BLOOD 309 (1995).
IL-1 receptor (linear)	inflammatory and autoimmune diseases ("IL-1 antagonist" or "IL-1 ra- mimetic")	U.S. Pat. No. 5,608,035; U.S. Pat. No. 5,786,331; U.S. Pat. No. 5,880,096; Yanofsky et al., 93 PNAS 7381-6 (1996); Akeson et al., 271 J. BIOL. CHEM. 30517-23 (1996); Wiekzorek et al., 49 POL. J. PHARMACOL. 107-17 (1997); Yanofsky, 93 PNAS 7381-7386 (1996).
Facteur thyrique (linear)	stimulation of lymphocytes (FTS-mimetic)	Inagaki-Ohara et al., 171 CELLULAR IMMUNOL. 30-40 (1996); Yoshida, 6 J. IMMUNOPHARMACOL 141-6 (1984).
CTLA4 MAb (intrapeptide di-sulfide bonded)	CTLA4-mimetic	Fukumoto et al., 16 NATURE BIOTECH. 267-70 (1998).
TNF-a receptor (exo-cyclic)	TNF-a antagonist	Takasaki et al., 15 NATURE BIOTECH. 1266-70 (1997); WO 98/53842, published December 3, 1998.
TNF-a receptor (linear)	TNF-a antagonist	Chirinos-Rojas, J. IMM., 5621-26.
C3b (intrapeptide di-sulfide)	inhibition of complement activation; autoimmune	Sahu et al., 157 IMMUNOL. 884-91 (1996); Morikis et al., 7 PROTEIN SCI. 619-27

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
bonded)	diseases (C3b antagonist)	(1998).
vinculin (linear)	cell adhesion processes, cell growth, differentiation wound healing, tumor metastasis ("vinculin binding")	Adey et al., 324 BIOCHEM. J. 523-8 (1997).
C4 binding protein (C413P) (linear)	anti-thrombotic	Linse et al. 272 BIOL. CHEM. 14658-65 (1997).
urokinase receptor (linear)	processes associated with urokinase interaction with its receptor (e.g. angiogenesis, tumor cell invasion and metastasis; (URK antagonist)	Goodson et al., 91 PNAS 7129-33 (1994); International patent application WO 97/35969, published October 2, 1997.
Mdm2, Hdm2 (linear)	Inhibition of inactivation of p53 mediated by Mdm2 or hdm2; anti-tumor ("Mdm/hdm antagonist")	Picksley et al., 9 ONCOGENE 2523-9 (1994); Bottger et al. 269 J. MOL. BIOL. 744-56 (1997); Bottger et al., 13 ONCOGENE 13: 2141-7 (1996).
p21 ^{WAF1} (linear)	anti-tumor by mimicking the activity of p21 ^{WAF1}	Ball et al., 7 CURR. BIOL. 71-80 (1997).
farnesyl transferase (linear)	anti-cancer by preventing activation of ras oncogene	Gibbs et al., 77 CELL 175-178 (1994).
Ras effector domain (linear)	anti-cancer by inhibiting biological function of the ras oncogene	Moodie et al., 10 TRENDS GENET. 44-48 (1994); Rodriguez et al., 370 NATURE 527-532 (1994).
SH2/SH3 domains (linear)	anti-cancer by inhibiting tumor growth with activated tyrosine kinases	Pawson et al., 3 CURR. BIOL. 434-432 (1993); Yu et al., 76 CELL 933-945 (1994).
p16 ^{INK4} (linear)	anti-cancer by mimicking activity of p16; e.g., inhibiting cyclin D-Cdk complex ("p16-mimetic")	Fahraeus et al., 6 CURR. BIOL. 84-91 (1996).
Src, Lyn (linear)	inhibition of Mast cell activation, IgE-related conditions, type I hypersensitivity ("Mast cell antagonist").	Stauffer et al., 36 BIOCHEM. 9388-94 (1997).
Mast cell protease (linear)	treatment of inflammatory disorders mediated by release of tryptase-6 ("Mast cell protease inhibitors")	International patent application WO 98/33812, published August 6, 1998.
SH3 domains (linear)	treatment of SH3-mediated disease states ("SH3 antagonist")	Rickles et al., 13 EMBO J. 5598-5604 (1994); Sparks et al., 269 J. BIOL. CHEM. 238536 (1994); Sparks et al., 93 PNAS 1540-44 (1996).
HBV core antigen (HBcAg) (linear)	treatment of HBV viral antigen (HBcAg) infections ("anti-HBV")	Dyson & Muray, PNAS 2194-98 (1995).
selectins (linear)	neutrophil adhesion inflammatory diseases	Martens et al., 270 J. BIOL. CHEM. 21129-36 (1995);

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
	("selectin antagonist")	European Pat. App. EP 0 714 912, published June 5, 1996.
calmodulin (linear, cyclized)	calmodulin antagonist	Pierce et al., 1 MOLEC. DRUGS 25965 (1995); Dedman et al., 267 J. BIOL. CHEM. 23025-30 (1993); Adey & Kay, 169 GENE 133-34 (1996).
integrins (linear, cyclized)	tumor-homing; treatment for conditions related to integrin-mediated cellular events, including platelet aggregation, thrombosis, wound healing, osteoporosis, tissue repair, angiogenesis (e.g., for treatment of cancer) and tumor invasion ("integrin-binding")	International patent applications WO 95/14714, published June 1, 1995; WO 97/08203, published March 6, 1997; WO 98/10795, published March 19, 1998; WO 99/24462, published May 20, 1999; Kraft et al., 274 J. BIOL. CHEM. 1979-85 (1999).
fibronectin and extracellular matrix components of T-cells and macrophages (cyclic, linear)	treatment of inflammatory and autoimmune conditions	International patent application WO 98/09985, published March 12, 1998.
somatostatin and cortistatin (linear)	treatment or prevention of hormone-producing tumors, acromegaly, gigantism, dementia, gastric ulcer, tumor growth, inhibition of hormone secretion, modulation of sleep or neural activity	European patent application EP 0 911 393, published Apr. 28, 1999.
bacterial lipopoly-saccharide (linear)	antibiotic; septic shock; disorders modulatable by CAP37	U.S. Pat. No. 5,877,151, issued March 2, 1999.
parlaxin, mellitin (linear or cyclic)	antipathogenic	International patent application WO 97/31019, published 28 August 1997.
VIP (linear, cyclic)	impotence, neuro-degenerative disorders	International patent application WO 97/40070, published October 30, 1997.
CTLs (linear)	cancer	European patent application EP 0 770 624, published May 2, 1997.
THF-gamma2 (linear)		Burnstein, 27 BIOCHEM. 4066-71 (1988).
Amylin (linear)		Cooper, 84 PNAS 8628-32 (1987).
Adreno-medullin (linear)		Kitamura, 192 BBRC 553-60 (1993).
VEGF (cyclic, linear)	anti-angiogenic; cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis ("VEGF antagonist")	Fairbrother, 37 BIOCHEM. 17754-64 (1998).
MMP	inflammation and	Koivunen, 17 NATURE BIOTECH. 768-74

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
(cyclic)	autoimmune disorders; tumor growth ("MMP inhibitor")	(1999).
HGH fragment (linear)		U.S. Pat. No. 5,869,452, issued Feb. 9, 1999.
Echistatin	inhibition of platelet aggregation	Gan, 263 J. BIOL. 19827-32 (1988).
SLE autoantibody (linear)	SLE	International patent application WO 96/30057, published Oct. 3, 1996.
GD1 alpha	suppression of tumor metastasis	Ishikawa et al., 1 FEBS LETT. 20-4 (1998).
anti-phospholipid β -2 glycoprotein-1 (β 2GPI) antibodies	endothelial cell activation, anti-phospholipid syndrome (APS), thromboembolic phenomena, thrombocytopenia, and recurrent fetal loss	Blank Mal., 96 PNAS 5164-8 (1999).
T-Cell Receptor β chain (linear)	diabetes	International patent application WO 96/101214, published Apr. 18, 1996.
Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
EPO receptor (intrapeptide disulfide-bonded)	EPO mimetic	Wrighton et al. (1996), Science 273: 458-63; U.S. Pat. No. 5,773,569, issued June 30, 1998 to Wrighton et al.
EPO receptor (C-terminally cross- linked dimer)	EPO mimetic	Livnah et al. (1996), Science 273: 464- 71; Wrighton et al. (1997), Nature Biotechnology 15:1261-5; int'l patent application WO 96/40772, published Dec. 19, 1996
EPO receptor (linear)	EPO mimetic	Naranda et al., 96 PNAS 7569-74 (1999)
c-Mpl (linear)	TPO-mimetic	Cwirla et al. (1997) Science 276:1696-9; U.S. Pat. No. 5,869,451, issued Feb. 9, 1999; U.S. Pat. No. 5,932,946, issued Aug. 3, 1999
c-Mpl (C-terminally cross- linked dimer)	TPO-mimetic	Cwirla et al. (1997) Science 276:1696-9
(disulfide-linked dimer)	stimulation of hematopoiesis ("G-CSF-mimetic")	Paukovits et al. (1984), Hoppe-Seylers Z. Physiol. Chem. 365: 30311; Laerum gal. (1988), Exp. Hemat. 16:274-80
(alkylene-linked dimer)	G-CSF-mimetic	Batnagar 91-al. (1996), linked dimer J. Med. Chem. 39:38149; Cuthbertson et al. (1997), J. Med. Chem. 40: 2876-82; King et al. (1991), Exp. Hematol. 19:481; King et al. (1995), Blood 86 (Suppl. 1): 309
IL-1 receptor (linear)	inflammatory and autoimmune diseases ("IL-1	U.S. Pat. No. 5,608,035; U.S. Pat. No. 5,786,331; U.S. Pat. No. 5,880,096;

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
	antagonist" or "IL-1 ra- mimetic")	Yanofsky 91-al. (1996) PNAS 93:7381-6; Akeson et al. (1996), J. Biol. Chem. 271: 30517-23; Wiekzorek et al. (1997), Pol. J. Pharmacol. 49:107-17; Yanofsky (1996), PNAs, 93:7381-7386.
Facteur thymique (linear)	stimulation of lymphocytes (FTS-mimetic)	Inagaki-Ohara et al. (1996), Cellular Immunol. 171: 30-40; Yoshida (1984), J. Immunopharmacol, 6:141-6.
CTLA4 MAb (intrapeptide di-sulfide bonded)	CTLA4-mimetic	Fukumoto et al. (1998), Nature Biotech. 16:267-70
TNF-a receptor (exo-cyclic)	TNF-a antagonist	Takasaki et al. (1997), Nature Biotech. 15:1266-70; WO 98/53842, published December 3, 1998.
TNF-a receptor (linear)	TNF-a antagonist	Chirinos-Rojas J. Imm., 5621-26.
C3b (intrapeptide di-sulfide bonded)	inhibition of complement activation; autoimmune diseases (C3b antagonist)	Sahu et al. (1996), Immunol. 157:884-91; Morikis et al. (1998), Protein Sci. 7:619- 27.
vinculin (linear)	cell adhesion processes, cell growth, differentiation wound healing, tumor metastasis ("vinculin binding")	Adey et al. (1997), Biochem. J. 324:523-8
C4 binding protein (C413P) (linear)	anti-thrombotic	Linse et al. 272 Biol. Chem. 14658-65 (1997)
urokinase receptor (linear)	processes associated with urokinase interaction with its receptor (e.g. angiogenesis, tumor cell invasion and metastasis; (URK antagonist)	Goodson et al. (1994), 91 PNAS 7129-33; International application WO 97/35969, published October 2, 1997
Mdm2, Hdm2 (linear)	Inhibition of inactivation of p53 mediated by Mdm2 or hdm2; anti-tumor ("Mdm/hdm antagonist")	Picksley et al. (1994), Oncogene 9: 2523- 9; Bottger et al. (1997) J. Mol. Biol. 269: 744-56; Bottger et al. (1996), Oncogene 13: 2141-7
p21 ^{WAF1} (linear)	anti-tumor by mimicking the activity of p21 ^{WAF1}	Ball et al.(1997), Curr. Biol. 7: 71-80.
farnesyl transferase (linear)	anti-cancer by preventing activation of ras oncogene	Gibbs et al. (1994), Cell 77:175-178
Ras effector domain (linear)	anti-cancer by inhibiting biological function of the ras oncogene	Moodie et at. (1994), Trends Genel 10:44- 48 Rodriguez et al. (1994), Nature 370:527-532 .
SH2/SH3 domains (linear)	anti-cancer by inhibiting tumor growth with activated tyrosine kinases	Pawson et al (1993), Curr. Biol. 3:434- 432, Yu et al. (1994), Cell 76:933-945.
p16 ^{INK4} (linear)	anti-cancer by mimicking activity of p16; e.g., inhibiting cyclin D-Cdk complex ("p,16-mimetic")	Fahraeus et al. (1996), Curr. Biol. 6:84-91
Src, Lyn	inhibition of Mast cell	Stauffer et al. (1997), Biochem. 36: 9388-

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
(linear)	activation, IgE-related conditions, type I hypersensitivity ("Mast cell antagonist").	94.
Mast cell protease (linear)	treatment of inflammatory disorders mediated by release of tryptase-6 ("Mast cell protease inhibitors")	International application WO 98/33812, published August 6, 1998
SH3 domains (linear)	treatment of SH3-mediated disease states ("SH3 antagonist")	Rickles et al. (1994), EMBO J. 13:5598-5604; Sparks et al. (1994), J. Biol. Chem. 269: 238536; Sparks et al. (1996), PNAS 93:1540-44.
HBV core antigen (HBcAg) (linear)	treatment of HBV viral antigen (HBcAg) infections ("anti-HBV")	Dyson & Muray (1995), Proc. Natl. Acad. Sci. 92:2194-98.
selectins (linear)	neutrophil adhesion inflammatory diseases ("selectin antagonist")	Martens et al. (1995), J. Biol. Chem. 270: 21129-36; European pat. app. EP 0 714 912, published June 5, 1996
calmodulin (linear, cyclized)	calmodulin antagonist	Pierce et al. (1995), Molec. Divemily 1: 25965; Dedman et al. (1993), J. Biol. Chem. 268: 23025-30; Adey & Kay (1996), Gene 169:133-34.
integrins (linear, cyclized)	tumor-homing; treatment for conditions related to integrin-mediated cellular events, including platelet aggregation, thrombosis, wound healing, osteoporosis, tissue repair, angiogenesis (e.g., for treatment of cancer) and tumor invasion ("integrin-binding")	International applications WO 95/14714, published June 1, 1995; WO 97/08203, published March 6, 1997; WO 98/10795, published March 19, 1998; WO 99/24462, published May 20, 1999; Kraft et al. (1999), J. Biol. Chem. 274:1979-85.
fibronectin and extracellular matrix components of T-cells and macrophages (cyclic, linear)	treatment of inflammatory and autoimmune conditions	WO 98/09985, published March 12, 1998.
somatostatin and cortistatin (linear)	treatment or prevention of hormone-producing tumors, acromegaly, gigantism, dementia, gastric ulcer, tumor growth, inhibition of hormone secretion, modulation of sleep or neural activity	European patent application 0 911 393, published Apr. 28, 1999.
bacterial lipopoly-saccharide (linear)	antibiotic; septic shock; disorders modulatable by CAP37	U.S. Pat. No. 5,877,151, issued March 2, 1999.

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
parclaxin, mellitin (linear or cyclic)	antipathogenic	WO 97/31019, published 28 August 1997.
VIP (linear, cyclic)	impotence, neuro- degenerative disorders	WO 97/40070, published October 30, 1997.
CTLs (linear)	cancer	EP 0 770 624, published May 2, 1997.
THF-gamma2 (linear)		Burnstein (1988), Biochem., 27:4066-71
Amylin (linear)		Cooper (1987), PNAS 84:8628-32.
Adreno-medullin (linear)		Kitamura (1993), BBRC, 192:553-60
VEGF (cyclic, linear)	anti-angiogenic; cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis ("VEGF antagonist")	Fairbrother (1998), Biochem., 37:17754-64.
MMP (cyclic)	inflammation and autoimmune disorders; tumor growth ("MMP inhibitor")	Koivunen 17 Nature Biotech., 768-74 (1999).
HGH fragment (linear)		U.S. Pat. No. 5,869,452.
Echistatin	inhibition of platelet aggregation	Gan (1988), J. Biol. 263:19827-32.
SLE autoantibody (linear)	SLE	WO 96/30057, published Oct. 3, 1996.
GD1 alpha	suppression of tumor metastasis	Ishikawa et al., 1 FEBS Lett. 20-4 (1998).
anti-phospholipid β -2 glycoprotein-1 (β 2GPI) antibodies	endothelial cell activation, anti-phospholipid syndrome (APS), thromboembolic phenomena, thrombocytopenia, and recurrent fetal loss	Blank Mal. (1999), PNAS 96: 5164-8.
T-Cell Receptor β chain (linear)	diabetes	WO 96/101214, published Apr. 18, 1996.

There are two pivotal cytokines in the pathogenesis of rheumatoid arthritis, IL-1 and TNF- α . They act synergistically to induce each other, other cytokines, and COX-2. Research suggests that IL-1 is a primary mediator of bone and cartilage destruction in

5 rheumatoid arthritis patients, whereas TNF- α appears to be the primary mediator of inflammation.

In a preferred embodiment of the invention, the pseudo-antibody comprises a target-binding moiety that binds to tumor necrosis factor alpha (TNF α), a pro-inflammatory cytokine. U.S. Patent No. 6,277,969, issued Aug. 21, 2001; U.S. Patent

No. 6,090,382, issued July 10, 2000. Anti-TNF α antibodies have shown great promise as therapeutics. For example, Infliximab, provided commercially as REMICADE® by Centocor, Inc. (Malvern, Penn.) has been used for the treatment of several chronic autoimmune diseases such as Crohn's disease and rheumatoid arthritis. Treacy, 19(4)
5 HUM. EXP. TOXICOL. 226-28 (2000); *see also* Chantry, 2(1) CURR. OPIN. ANTI-INFLAMMATORY IMMUNOMODULATORY INVEST. DRUGS 31-34 (2000); Rankin et al., 34(4) BRIT. J. RHEUMATOLOGY 334-42 (1995). Preferably, any exposed amino acids of the TNF α -binding moiety of the pseudo-antibody are those with minimal antigenicity in humans, such as human or humanized amino acid sequences. These moieties may be
10 generated by screening libraries, as described above, by grafting human amino acid sequences onto murine-derived paratopes (Siegel et al., 7(1) CYTOKINE 15-25 (1995); WO 92/11383, published July 9, 1992) or monkey-derived paratopes (WO 93/02108, published Feb. 4, 1993), or by utilizing xenomice (WO 96/34096, published Oct. 31, 1996). Alternatively, murine-derived anti-TNF α antibodies have exhibited efficacy.
15 Saravolatz et al., 169(1) J. INFECT. DIS. 214-17 (1994).

Alternatively, instead of being derived from an antibody, the TNF α binding moiety of the pseudo-antibody may be derived from the TNF α receptor. For example, Etanercept is a recombinant, soluble TNF α receptor molecule that is administered subcutaneously and binds to TNF α in the patient's serum, rendering it biologically
20 inactive. Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of etanercept contains the C_H2 domain, the C_H3 domain and hinge region, but not the C_H1 domain of IgG1. Etanercept is produced by recombinant DNA technology in a Chinese hamster
25 ovary (CHO) mammalian cell expression system. It consists of 934 amino acids and has an apparent molecular weight of approximately 150 kilodaltons. Etanercept may be obtained as ENBREL™, manufactured by Immunex Corp. (Seattle, Wash.). Etanercept may be efficacious in rheumatoid arthritis. Hughes et al., 15(6) BIODRUGS 379-93 (2001).

30 Another form of human TNF receptor exists as well, identified as p55. Kalinkovich et al., J. INTERFERON & CYTOKINE RES. 15749-57 (1995). This receptor has also been explored for use in therapy. *See, e.g.,* Qian et al. 118 ARCH. OPHTHALMOL.

1666-71 (2000). A previous formulation of the soluble p55 TNF receptor had been coupled to polyethylene glycol [r-metHuTNFbp PEGylated dimer (TNFbp)], and demonstrated clinical efficacy but was not suitable for a chronic indication due to the development antibodies upon multiple dosing, which resulted in increased clearance of the drug. A second generation molecule was designed to remove the antigenic epitopes of TNFbp, and may be useful in treating patients with rheumatoid arthritis. Davis et al., Presented at the Ann. European Cong. Rheumatology, Nice, France (June 21-24, 2000).

IL-1 receptor antagonist (IL-1Ra) is a naturally occurring cytokine antagonist that demonstrates anti-inflammatory properties by balancing the destructive effects of IL-1 α and IL-1 β in rheumatoid arthritis but does not induce any intracellular response. Hence, in a preferred embodiment of the invention, the pseudo-antibody comprises IL-1Ra, or any structural or functional analog thereof. Two structural variants of IL-1Ra exist: a 17-kDa form that is secreted from monocytes, macrophages, neutrophils, and other cells (sIL-1Ra) and an 18-kDa form that remains in the cytoplasm of keratinocytes and other epithelial cells, monocytes, and fibroblasts (icIL-1Ra). An additional 16-kDa intracellular isoform of IL-1Ra exists in neutrophils, monocytes, and hepatic cells. Both of the major isoforms of IL-1Ra are transcribed from the same gene through the use of alternative first exons. The production of IL-1Ra is stimulated by many substances including adherent IgG, other cytokines, and bacterial or viral components. The tissue distribution of IL-1Ra in mice indicates that sIL-1Ra is found predominantly in peripheral blood cells, lungs, spleen, and liver, while icIL-1Ra is found in large amounts in skin. Studies in transgenic and knockout mice indicate that IL-1Ra is important in host defense against endotoxin-induced injury. IL-1Ra is produced by hepatic cells with the characteristics of an acute phase protein. Endogenous IL-1Ra is produced in human autoimmune and chronic inflammatory diseases. The use of neutralizing anti-IL-1Ra antibodies has demonstrated that endogenous IL-1Ra is an important natural antiinflammatory protein in arthritis, colitis, and granulomatous pulmonary disease. Patients with rheumatoid arthritis treated with IL-1Ra for six months exhibited improvements in clinical parameters and in radiographic evidence of joint damage. Arend et al., 16 ANN. REV. IMMUNOL. 27-55 (1998).

Yet another example of an IL-1Ra that may be incorporated into the pseudo-antibody of the present invention is a recombinant human version called interleukin-1

17.3 Kd met-IL1ra, or Anakinra, produced by Amgen, (San Francisco, Cal.) under the name KINERET™. Anakinra has also shown promise in clinical studies involving patients with rheumatoid arthritis. Presented at the 65th Ann. Sci. Meeting of Am. College Rheumatology (Nov. 12, 2001).

- 5 Another embodiment of the pseudo-antibody includes a moiety that targets cyclooxygenase-2 (COX-2). COX-2 selective inhibitors-such as valdecoxib, etoricoxib, celecoxib and rofecoxib are less toxic to the gastrointestinal (GI) tract than conventional nonsteroidal anti-inflammatory drugs (NSAIDs), while possessing equivalent analgesic efficacy for conditions such as osteoarthritis (OA), rheumatoid
10 arthritis (RA), dental pain and menstrual pain. In a preferred embodiment of the invention, a COX-2 inhibitor may be included in the pseudo-antibody construct with a TNF α antagonist. *See, e.g.*, U.S. Patent Nos. 5,474,995, 5,409,944.

- In another embodiment of the invention, the pseudo-antibody includes a selective p38 Mitogen-Activated Protein Kinase (p38 MAP kinase) inhibitor. For
15 example, the compound SB 242235 is a potent and selective p38 MAP kinase inhibitor. The compound is active in the adjuvant arthritic rat, where it inhibits inflammation and has significant joint-protective effects as measured by changes in bone mineral density, magnetic resonance imaging, micro-computed tomography, and histology. These studies indicate that cytokine-suppressing, low molecular weight p38 inhibitors may be
20 orally active, disease-modifying agents in the treatment of rheumatoid arthritis. Badger et al, *Disease-Modifying Activity of SB 242235, A Selective Inhibitor of p38 Mitogen-Activated Protein Kinase, in Rat Adjuvant-Induced Arthritis*, Proceedings of the 1999 AACR, NCI, EORTC Int'l Conference, Am. Assoc. for Cancer Res.

- In another embodiment of the invention, the pseudo-antibody comprises a
25 target-binding moiety that binds interleukin 12 (IL-12), a heterodimeric cytokine consisting of glycosylated polypeptide chains of 35 and 40 kD which are disulfide bonded. The cytokine is synthesized and secreted by antigen presenting cells, including dendritic cells, monocytes, macrophages, B cells, Langerhans cells and keratinocytes, as well as natural killer (NK) cells. IL-12 mediates a variety of biological processes
30 and has been referred to as NK cell stimulatory factor (NKSF), T-cell stimulating factor, cytotoxic T-lymphocyte maturation factor and EBV-transformed B-cell line factor. Curfs et al., 10 CLIN. MICRO. REV. 742-80 (1997). Interleukin-12 can bind to the IL-12 receptor expressed on the plasma membrane of cells (e.g., T cells, NK cell),

thereby altering (e.g., initiating, preventing) biological processes. For example, the binding of IL-12 to the IL-12 receptor can stimulate the proliferation of pre-activated T cells and NK cells, enhance the cytolytic activity of cytotoxic T cells (CTL), NK cells and LAK (lymphokine activated killer) cells, induce production of gamma interferon (IFN GAMMA) by T cells and NK cells and induce differentiation of naive Th0 cells into Th1 cells that produce IFN GAMMA and IL-2. Trinchieri, 13 ANN. REV. IMMUNOLOGY 251-76 (1995). In particular, IL-12 is vital for the generation of cytolytic cells (e.g., NK, CTL) and for mounting a cellular immune response (e.g., a Th1 cell mediated immune response). Thus, IL-12 is critically important in the generation and regulation of both protective immunity (e.g., eradication of infections) and pathological immune responses (e.g., autoimmunity). Hendrzak et al., 72 LAB. INVESTIGATION 619-37 (1995). Accordingly, an immune response (e.g., protective or pathogenic) can be enhanced, suppressed or prevented by manipulation of the biological activity of IL-12 *in vivo*, for example, by means of an antibody.

In another embodiment of the present invention, the pseudo-antibody comprises or targets an integrin. Integrins have been implicated in the angiogenic process, by which tumor cells form new blood vessels that provide tumors with nutrients and oxygen, carry away waste products, and to act as conduits for the metastasis of tumor cells to distant sites, Gastl et al., 54 ONCOL. 177-84 (1997). Integrins are heterodimeric transmembrane proteins that play critical roles in cell adhesion to the extracellular matrix (ECM) which, in turn, mediates cell survival, proliferation and migration through intracellular signaling. During angiogenesis, a number of integrins that are expressed on the surface of activated endothelial cells regulate critical adhesive interactions with a variety of ECM proteins to regulate distinct biological events such as cell migration, proliferation and differentiation. Specifically, the closely related but distinct integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ have been shown to mediate independent pathways in the angiogenic process. An antibody generated against $\alpha V\beta 3$ blocked basic fibroblast growth factor (bFGF) induced angiogenesis, whereas an antibody specific to $\alpha V\beta 5$ inhibited vascular endothelial growth factor-induced (VEGF-induced) angiogenesis. Eliceiri et al., 103 J. CLIN. INVEST. 1227-30 (1999); Friedlander et al., 270 SCIENCE 1500-02 (1995).

In another preferred embodiment of the invention, the pseudo-antibody comprises at least one glycoprotein IIb/IIIa receptor antagonist. More specifically, the

final obligatory step in platelet aggregation is the binding of fibrinogen to an activated membrane-bound glycoprotein complex, GP IIb/IIIa. Platelet activators such as thrombin, collagen, epinephrine or ADP, are generated as an outgrowth of tissue damage. During activation, GP IIb/IIIa undergoes changes in conformation that results in exposure of occult binding sites for fibrinogen. There are six putative recognition sites within fibrinogen for GP IIb/IIIa and thus fibrinogen can potentially act as a hexavalent ligand to crossing GP IIb/IIIa molecules on adjacent platelets. A deficiency in either fibrinogen or GP IIb/IIIa prevents normal platelet aggregation regardless of the agonist used to activate the platelets. Since the binding of fibrinogen to its platelet receptor is an obligatory component of normal aggregation, GP IIb/IIIa is an attractive target for an antithrombotic agent.

Results from clinical trials of GP IIb/IIIa inhibitors support this hypothesis. A Fab fragment of the monoclonal antibody 7E3, which blocks the GP IIb/IIIa receptor, has been shown to be an effective therapy for the high risk angioplasty population. It is used as an adjunct to percutaneous transluminal coronary angioplasty or atherectomy for the prevention of acute cardiac ischemic complications in patients at high risk for abrupt closure of the treated coronary vessel. Although 7E3 blocks both the IIb/IIIa receptor and the $\alpha_v\beta_3$ receptor, its ability to inhibit platelet aggregation has been attributed to its function as a IIb/IIIa receptor binding inhibitor. The IIb/IIIa receptor antagonist may be, but is not limited to, an antibody, a fragment of an antibody, a peptide, or an organic molecule. For example, the target-binding moiety may be derived from 7E3, an antibody with glycoprotein IIb/IIIa receptor antagonist activity. 7E3 is the parent antibody of c7E3, a Fab fragment known as abciximab, known commercially as REOPRO® produced by Centocor, Inc. (Malvern, Penn.). Abciximab binds and inhibits the adhesive receptors GPIIb/IIIa and $\alpha_v\beta_3$, leading to inhibition of platelet aggregation and thrombin generation, and the subsequent prevention of thrombus formation. U.S. Patent Nos. 5,976,532, 5,877,006, 5,770,198; Coller, 78 THROM HAEMOST. 730-35 (1997); JORDAN ET AL., in ADHESION RECEPTORS AS THERAPEUTIC TARGETS 281-305 (Horton, ed. CRC Press, New York, 1996); Jordan et al., in NEW THERAPEUTIC AGENTS IN THROMBOSIS & THROMBOLYSIS (Sasahara & Loscalzo, eds. Marcel Dekker, Inc. New York, 1997).

Additionally, the glycoprotein IIb/IIIa receptor antagonist of the present invention may further comprise a thrombolytic. For example, the thrombolytic may be

tPA, or a functional variation thereof. RETAVASE®, produced by Centocor, Inc. (Malvern, Penn.), is a variant tPA with a prolonged half-life. In mice, the combination of Retavase and the IIb/IIIa receptor antagonist c7E3 Fab markedly augmented the dissolution of pulmonary embolism. See Provisional Patent Application Serial
5 No. 60/304409.

Alternative target-binding moieties envisioned in the present invention also include non-peptide molecules. For example, tirofiban hydrochloride is a non-peptide antagonist of the platelet glycoprotein IIb/IIIa receptor, that inhibits platelet aggregation. See U.S. Patent No. 6,117,842, issued Sept. 12, 2000. Tirofiban is
10 commercially available as AGGRASTAT® from Merck & Co., Inc., (Whitehouse Station, N.J.), manufactured by Baxter Healthcare Corp. (Deerfield, Ill.) and Ben Venue Labs. (Bedford, Ohio). Tirofiban has the structure illustrated in Example 10, Structure 2, and has an *in vivo* circulatory half-life of approximately two hours. The pseudo-antibody is created by attaching an additional moiety to an aromatic site on the
15 molecule, such that the additional moiety (depicted as "X" in Structure 2), is or contains a functional group capable of forming the pseudo-antibody structure, as long as some activity of the parent compound is retained.

Other examples of non-peptide target binding moieties that may be included in the pseudo-antibodies of the present invention include leflunomide (ARAVA™), which
20 has the chemical name α,α,α -Trifluoro-5-methyl-4-isoxazolecarboxy-p-toluidide. Leflunomide is a prodrug which is changed in the body to an active metabolite. An immuno-suppressive agent, it inhibits pyrimidine synthesis and thus reduces the production of immune cells that attack joints, and may be useful for relief of the signs and symptoms
25 of arthritis.

In another embodiment of the instant invention, the pseudo-antibody construct includes a moiety that inhibits matrix metalloproteases (MMPs). MMPs are involved in invasion, metastasis and angiogenesis. MMPs 2 & 9 are overexpressed in the tumor/stroma of multiple cancers, and are thus attractive targets for inhibition.
30 BAY12-9566 is a selective, non-peptidic biphenyl inhibitor of MMPs (MMPI), exhibiting nM inhibitory activity against MMPs 2, 3 & 9 with anti-invasive, anti-metastatic and anti-angiogenic activity in preclinical models and clinical evaluations in human patients. Lathia et al., Proc. 1999 AACR, NCI, EORTC Int'l Conf., Am. Assoc.

Cancer Res. MMPs, often thought of as promising anti-cancer therapeutics, are also being investigated for use in rheumatoid arthritis therapy. Other MMPs include Marimastat and BB-2983. *See, e.g.*, Boasberg et al., 15 Proc. Ann. Meeting Am. Soc. Clin. Oncol. A671 (1996).

5 The pseudo-antibodies of the present invention also include moieties such as receptors, or fragments thereof, and activated receptors, i.e., peptides associated with their corresponding receptors, or fragments thereof. These complexes may mimic activated receptors and thus affect a particular biological activity. Alternatively, the receptor can be genetically re-engineered to adopt the activated conformation. For
10 example, the thrombin-bound conformation of fibrinopeptide A exhibits a strand-turn-strand motif, with a β -turn centered at residues Glu-11 and Gly-12. Molecular modeling analysis indicates that the published fibrinopeptide conformation cannot bind reasonably to thrombin, but that reorientation of two residues by alignment with bovine pancreatic trypsin inhibitor provides a good fit within the deep thrombin cleft and
15 satisfies all of the experimental nuclear Overhauser effect data. Based on this analysis, a researchers were able to successfully design and synthesize hybrid peptide mimetic substrates and inhibitors that mimic the proposed β -turn structure. The results indicate that the turn conformation is an important aspect of thrombin specificity, and that the turn mimetic design successfully mimics the thrombin-bound conformation of
20 fibrinopeptide. Nakanishi et al., 89(5) PNAS 1705-09 (1992).

 Another example of activated-receptor moieties concerns the peptido mimetics of the erythropoietin (Epo) receptor. By way of background, the binding of Epo to the Epo receptor (EpoR) is crucial for production of mature red blood cells. The Epo-bound, activated EpoR is a dimer. *See, e.g.*, Constantinescu et al., 98 PNAS 4379-84
25 (2001). In its natural state, the first EpoR in the dimer binds Epo with a high affinity whereas the second EpoR molecule binds to the complex with a low affinity. Bivalent anti-EpoR antibodies have been reported to activate EopR, probably by dimerization of the EpoR. Additionally, small synthetic peptides, that do not have any sequence homology with the Epo molecule, are also able to mimic the biologic effects of Epo but
30 with a lower affinity. Their mechanism of action is probably also based on the capacity to produce dimerization of the EpoR. Hence, an embodiment of the present invention provides for a pseudo-antibody comprising an activated EpoR mimetic.

In another preferred embodiment of the invention, the pseudo-antibody may include antimicrobial agents or portions thereof, which include antibacterial agents, antiviral agents, antifungal agents, antimycobacterial agents, and antiparasitic agents. Antibacterials include, but are not limited to, Beta-lactams (such as Penicillins and Cephalsporins), Aminoglycosides (such as Gentamicin), Macrolides (such as Erythromycin), Fluoroquinolones, Metronidazole, Sulfonamides, Tetracyclines, Trimethoprim, and Vancomycin. Antifungal agents include, but are not limited to Amphotericin, Fluconazole, Flucytosine, Itraconazole, and Ketoconazole. Antiparasitic agents include, but are not limited to, Ivermectin, Mebendazole, Mefloquine, Pentamidine, Praziquantel, Pyrimethamine, and Quinine. Antiviral agents include, but are not limited to, Acyclovir, Amantadine, Didanosine, Famciclovir, Foscarnet, Ganciclovir, Rimatandine, Stavudine, Zalcitabine, and Zidovudine. Antimycobacterial agents include, but are not limited to, Isoniazid, Rifampin, Streptomycin, Dapsone. SANFORD ET AL., GUIDE TO ANTIMICROBIAL THERAPY (25th ed., Antimicrobial Therapy, Inc., Dallas, Tex. 1995).

In another embodiment of the invention, the pseudo-antibody targets a cell cycle protein. In yet another embodiment of the invention, the pseudo-antibody includes a cell cycle protein, or a functionally active portion of a cell cycle protein. These cell cycle proteins are known in the art, and include cyclins, such as G₁ cyclins, S-phase cyclins, M-phase cyclins, cyclin A, cyclin D and cyclin E; the cyclin-dependent kinases (CDKs), such as G₁ CDKs, S-phase CDKs and M-phase CDKs, CDK2, CDK4 and CDK 6; and the tumor suppressor genes such as Rb and p53. Cell cycle proteins also include those involved in apoptosis, such as Bcl-2 and caspase proteins; proteins associated with Cdc42 signaling, p70 S6 kinase and PAK regulation; and integrins, discussed elsewhere. Also included in the cell cycle proteins of the present invention are anaphase-promoting complex (APC) and other proteolytic enzymes. The APC triggers the events leading to destruction of the cohesins and thus allowing sister chromatids to separate, and degrades the mitotic (M-phase) cyclins. Other relevant cell cycle proteins include S-phase promoting factor, M-phase promoting factor that activates APC. Kimball, *Kimball's Biology Pages*, at <http://www.ultranet.com/~jkimball/BiologyPages>.

The pseudo-antibody of the present invention may also incorporate or target a particular antigen. Antigens, in a broad sense, may include any molecule to which an

antibody, or functional fragment thereof, binds. Such antigens may be pathogen derived, and be associated with either MHC class I or MHC class II reactions. These antigens may be proteinaceous or include carbohydrates, such as polysaccharides, glycoproteins, or lipids. Carbohydrate and lipid antigens are present on cell surfaces of all types of cells, including normal human blood cells and foreign, bacterial cell walls or viral membranes. Nucleic acids may also be antigenic when associated with proteins, and are hence included within the scope of antigens encompassed in the present invention. See SEARS, IMMUNOLOGY (W. H. Freeman & Co. and Sumanas, Inc., 1997), available on-line at <http://www.whfreeman.com/immunology>.

For example, antigens may be derived from a pathogen, such as a virus, bacterium, mycoplasma, fungus, parasite, or from another foreign substance, such as a toxin. Such bacterial antigens may include or be derived from *Bacillus anthracis*, *Bacillus tetani*, *Bordetella pertussis*, *Brucella* spp., *Corynebacterium diphtheriae*, *Clostridium botulinum*, *Clostridium perfringens*, *Coxiella burnetii*, *Francisella tularensis*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, *Shigella* spp., *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Treponema pallidum*, *Yersinia pestis*, *Vibrio cholerae*. Often, the oligosaccharide structures of the outer cell walls of these microbes afford superior protective immunity, but must be conjugated to an appropriate carrier for that effect.

Viruses and viral antigens that are within the scope of the current invention include, but are not limited to, HBeAg, Hepatitis B Core, Hepatitis B Surface Antigen, Cytomegalovirus B, HIV-1 gag, HIV-1 nef, HIV-1 env, HIV-1 gp41-1, HIV-1 p24, HIV-1 MN gp120, HIV-2 env, HIV-2 gp 36, HCV Core, HCV NS4, HCV NS3, HCV p22 nucleocapsid, HPV L1 capsid, HSV-1 gD, HSV-1 gG, HSV-2 gG, HSV-II, Influenza A (H1N1), Influenza A (H3N2), Influenza B, Parainfluenza Virus Type 1, Epstein Barr virus capsid antigen, Epstein Barr virus, *Poxviridae Variola major*, *Poxviridae Variola minor*, Rotavirus, Rubella virus, Respiratory Syncytial Virus, Surface Antigens of the *Syphilis* spirochete, Mumps Virus Antigen, *Varicella zoster* Virus Antigen and *Filoviridae*.

Other parasitic pathogens such as *Chlamydia trachomatis*, *Plasmodium falciparum*, and *Toxoplasma gondii* may also provide antigens for, or be targeted by, the pseudo-antibody of the present invention. Numerous bacterial and viral, and other

microbe-generated antigens are available from commercial suppliers such as Research Diagnostics, Inc. (Flanders, N.J.).

Toxins, toxoids, or antigenic portions of either, within the scope of the present invention include those produced by bacteria, such as diptheria toxin, tetanus toxin, botulin toxin and enterotoxin B; those produced by plants, such as Ricin toxin from the castor bean *Ricinus communis*. Mycotoxins, produced by fungi, that may serve in the present invention include diacetoxyscirpenol (DAS), Nivalenol, 4-Deoxynivalenol (DON), and T-2 Toxin. Other toxins and toxoids produced by or derived from other plants, snakes, fish, frogs, spiders, scorpions, blue-green algae, snails may also be incorporated in the pseudo-antibody constructs of the present invention.

A use of antigen constructs can be as immunogens to elicit an immune response in animals for the generation of antibodies or as synthetic vaccines in man to elicit a protective immune response.

Antigens included in the pseudo-antibody constructs of the present invention may also serve as markers for particular cell types, or as targets for an agent interacting with that cell type. Examples include Human Leukocyte Antigens (HLA markers), MHC Class I and Class II, the numerous CD markers useful for identifying T-cells and the physiological states thereof. Alternatively, antigens may serve as "markers" for a particular disease or condition, or as targets of a therapeutic agent. Examples include, Prostate Specific Antigen, Pregnancy specific beta 1 glycoprotein (SP1), Thyroid Microsomal Antigen, and Urine Protein 1. Antigens may include those defined as "self" implicated in autoimmune diseases. Haptens, low molecular weight compounds such as drugs or antibiotics that are too small to cause an immune response unless they are coupled with much larger entities, may serve as antigens when coupled to the pseudo-antibody of the present invention. See ROITT ET AL., IMMUNOLOGY (5th ed., 1998); BENJAMINI ET AL., IMMUNOLOGY, A SHORT COURSE (3rd ed., 1996).

The pseudo-antibody of the present invention may also include an organic moiety to which, through the optional use of a linker, the target-binding moiety is attached. The organic moiety serves to position the target-binding moiety to optimize avidity, affinity, and/or circulating half-life. This moiety can be a hydrophilic polymeric group, a simple or complex carbohydrate, a fatty acid group, a fatty acid ester group, a lipid group, or a phospholipid group. More specifically, polyglycols are hydrophilic polymers that have one or more terminal hydroxy groups, such as

polyethylene glycol, polypropylene glycol, polyvinyl pyrrolidone, homo-polyamino acids, hetero-polyamino acids, and polyamides. In particular embodiments, the hydrophilic polymeric group can have a molecular weight of about 800 to about 120,000 Daltons and can be a polyalkane glycol (e.g., polyethylene glycol (PEG),
5 polypropylene glycol (PPG)), carbohydrate polymer, amino acid polymer or polyvinyl pyrrolidone, and the fatty acid or fatty acid ester group can comprise from about eight to about forty carbon atoms.

PEG is a generic name for mixtures of condensation polymers of ethylene oxide and water, represented by the general formula $H(OCH_2CH_2)_n OH$, in which n is greater
10 or equal to 4. Those PEGs with an average molecular weight of about 200 to 700 are liquid, and those above 1000 are waxlike solids. PEGs can be esterified with fatty acids to produce non-ionic surfactants in which the PEG functions as the hydrophile. PEGs increase the water solubility of a final product. Higher molecular PEGs impart a greater degree of water solubility than lower molecular weight PEGs.

15 PPGs are water soluble at low molecular weights (P425), but most PPGs are considered sparingly soluble in water. The secondary hydroxy group of polypropylene glycols is not as reactive as the primary hydroxy group on PEGs.

The pseudo-antibodies of the invention comprise at least one target-binding moiety bound to an organic moiety. In the instance in which the target-binding moiety
20 is an antibody, the organic moiety may be covalently bonded to a carboxyl-terminus of the antibody and/or covalently bonded to the sulfur atom of a cysteinyl residue of the antibody and/or attached by other site-specific methodology such as enzyme-catalyzed transamidation. Thus, the invention provides antibodies comprising site-specific modifications. For example, a modified Fab of an IgG can comprise a PEG moiety,
25 which is bonded to the carboxyl-terminus of the heavy chain. In another embodiment, several modified Fab' fragments are each bonded to a PEG molecule by sulfur atom of one of the cysteinyl residues that are contained within the hinge region of the heavy chain (the cysteine residues in the hinge region which form inter-chain disulfide bonds in the corresponding IgG or F(ab)). In yet another embodiment, at least two modified
30 Fab fragments, generated through the action of achromopeptidase, are bonded to one PEG moiety at the carboxyl-terminus of the heavy chain.

Attachment of the hydrophilic polymer can be by non-site specific means, under conditions that do not adversely affect the activity of the target-binding moiety,

although site-specific attachment is preferred. Examples of methods of attachment include, but are not limited to: (a) Glyoxyl modification of a N-terminal amino group followed by reductive alkylation with an amine, hydrazine, oxime, semicarbazide, or other appropriate nucleophile; (b) Periodic acid oxidation of one or more carbohydrates on a moiety, followed by reductive alkylation with an amine, hydrazine, oxime, semicarbazide, or other nucleophile; (c) Reverse proteolysis to attach an organic moiety containing a nucleophile to the C- or N- termini of a peptide, followed by reductive alkylation, or reaction with a suitable electrophile; and (d) Production of a recombinant peptide containing one or more additional cysteines, followed by its reaction with a suitable maleamide to form a thioether or activated thiol to form a disulfide, or halo compound to form a thioether. Other methods that may be employed are known to those of ordinary skill in the art. See LUNDBLAD, TECHNIQUES IN PROTEIN MODIFICATION (CRC Press, 1995). A specific example of N-terminal derivatization of EPO with an unfunctionalized PEG is discussed in U.S. Pat. No. 6,077,939. See also WO 00/26256, published May 11, 2000.

Additionally, in another embodiment of the invention, an additional organic molecule is included in the pseudo-antibody construct. This additional organic molecule is selected from the group consisting of fatty acids, dicarboxylic acids, monoesters or monoamides of dicarboxylic acids, lipids containing saturated fatty acids, lipids containing unsaturated fatty acids, lipids containing mixtures of unsaturated fatty acids, simple carbohydrates, complex carbohydrates, carbocycles (such as steroids), heterocycles (such as alkaloids), amino acid chains, proteins, enzymes, enzyme cofactors, and vitamins. In yet another embodiment of the invention, the additional organic molecule is a lipid. In a yet another preferred embodiment of the invention, this molecule is distearylphosphatidyl-ethanolamine (DSPE).

As noted previously, the pseudo-antibody of the present invention may affect a specific ligand, such as but not limited to where such pseudo-antibody modulates, decreases, increases, antagonizes, agonizes, mitigates, alleviates, blocks, inhibits, abrogates and/or interferes with at least one biological molecule's activity or binding, or with a receptor activity or binding, *in vitro*, *in situ* and/or *in vivo*. The pseudo-antibodies of the present invention can be used to measure or effect in an cell, tissue, organ or animal (including mammals and humans), to diagnose, monitor, modulate, treat, alleviate, help prevent the incidence of, or reduce the symptoms of, at least one

condition. In particular, the pseudo-antibody constructs may be used: to treat stenosis and/or restenosis following a vascular intervention; to prevent ischemia; to inhibit the growth and/or metastasis of a tumor; to inhibit a biological process mediated by the binding of a ligand to either or both of GPIIb/IIIa and $\alpha_v\beta_3$, expressed on the plasma
5 membrane of a cell; and to inhibit angiogenesis. Such a method can comprise administering an effective amount of a composition or a pharmaceutical composition comprising at least one pseudo-antibody to a cell, tissue, organ, animal or patient in need of such modulation, treatment, alleviation, prevention, or reduction in symptoms, effects or mechanisms. The effective amount can comprise an amount of
10 about 0.001 mg/kg to 500 mg/kg per single (e.g., bolus), multiple or continuous administration, or to achieve a serum concentration of 0.01-5000 $\mu\text{g/ml}$ serum concentration per single, multiple, or continuous administration, or any effective range or value therein, as done and determined using known methods, as described herein or known in the relevant arts.

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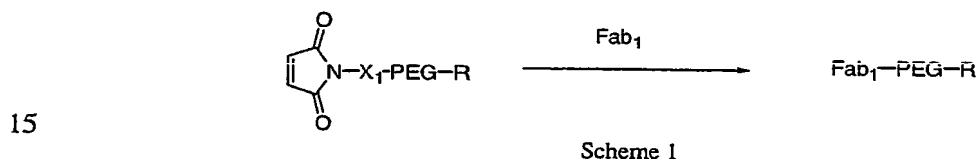
EXAMPLES

Certain constructs described herein may be similar to previously disclosed compounds, such as a Fab' antibody fragment with two PEG chains. WO 0026256; published May 11, 2000. The descriptions herein are not meant to be exclusive of all
20 previously disclosed compounds but are meant to define the broadest scope of this concept.

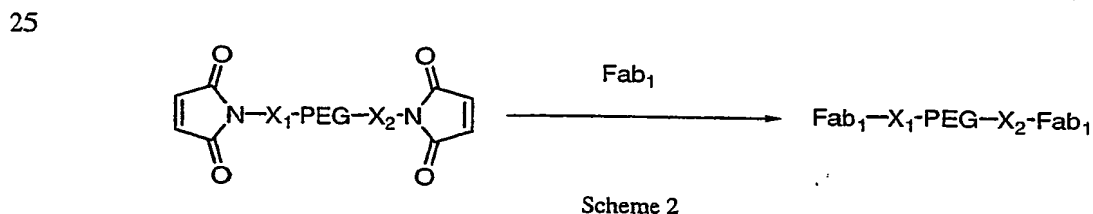
For purposes of illustrating the scope of the invention, a Fab molecule is used in pseudo-antibody (ΨAb) constructs. The use of this example is not meant to limit the scope of the invention to antibody fragments. The Fab contains a single free thiol (an
25 SH group) in the form of a cysteine, located toward or on the C-terminus of the heavy or light chain. By analogy, a single chain antibody, peptide, or organic molecule with a free thiol could also be used. While the method of constructing the example ΨAbs uses the spontaneous reaction of a thiol with a maleimide, other methods of covalent bond formation are envisioned as well. Examples, not meant to limit or define the scope of
30 the invention disclosure, include the spontaneous reaction of azides with trivalent phosphorus species such as dimethoxy-alkylphosphites to form phosphoramidates, the reductive alkylation of carbonyl compounds with amine derivatives and the spontaneous reaction of thiols with bromoacetyl derivatives to form thioethers.

Example 1.

Construct 1, shown in scheme 1, illustrates the addition of a single Fab to a maleimido-PEG, where the molecular weight of the PEG is such that the construct has a longer *in vivo* half-life than Fab₁, R can be an alkoxy group such as methoxyl or a compound selected from the structural categories of carbohydrates, saturated or unsaturated mono- or di-carboxylic acids, monoesters or amides of saturated or unsaturated di-carboxylic acids, higher alkoxy groups, lipids or other biologically compatible organic molecules. X₁ is an optional linker or spacer between the maleimide moiety and the PEG. The preferred method of synthesis for these constructs is shown in Scheme 1, where the R group has been previously attached to the PEG; however, synthetic schemes can be envisioned where the R group is attached to the PEG after the Fab-maleimide reaction. Additional activity can be imparted to these constructs by the R group.

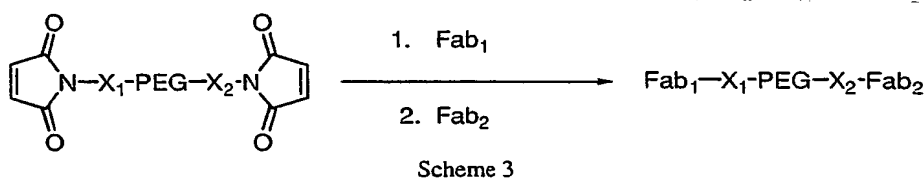
**Example 2.**

Construct 2, shown in Scheme 2, has identical Fabs on opposite ends of a PEG where the molecular weight of the PEG is such that the construct has a longer *in vivo* half-life than Fab₁. X₁ and X₂ are linkers between the PEG and the maleimide groups and may be either structurally identical or structurally unique. This type of construct has the advantage over an IgG in that the two Fabs can bind to identical receptors that are significantly further apart than could be bridged by a conventional immunoglobulin.

**Example 3.**

Construct 3, shown in Scheme 3, is composed of different Fabs on opposite ends of a PEG where the molecular weight of the PEG is such that the construct has a longer *in vivo* half-life than the Fabs from which it is constructed. This type of

bifunctional Ψ Ab construct has the advantage over a conventional bifunctional antibody fragment in that the two Fabs can bind to non-identical receptors that are significantly further apart than could be bridged by a conventional bifunctional construct. The synthesis of this type of construct is illustrated using sequential addition of the Fabs to a bis-maleimido-PEG, although other synthetic routes can be envisioned as well. This type of construct is well suited to a synthetic route in which the chemistry of attachment of the two Fabs is different, or the addition of one maleimide to the PEG is done after the addition of the first Fab.



Example 4.

15 Construct 4, shown in Scheme 4, has two identical Fabs on the same end of a PEG, where Q can be an alkoxy group such as methoxyl or a compound selected from the structural categories of carbohydrates, saturated or unsaturated mono- or di-carboxylic acids, monoesters or amides of saturated or unsaturated di-carboxylic acids, higher alkoxy groups, lipids or other biologically compatible organic molecules. When the Fab moiety has a single free -SH group, maleimide is used. In a preferred embodiment, Q is diesteroylphosphatidylethanolamine. Q can be also be an active molecule such as a toxin or a radioisotope, or a marker such as GFP. Y₁ and Z₁ are linkers or spacers between the maleimide moiety and the PEG and can be the same or different. W is a trifunctional moiety such that one functionality can be attached to a PEG and the other two can be attached to the linkers Y₁ and Z₁. As an example, Q is methoxyl, PEG is NH₂-PEG, W₁ is Lysine, and Y₁ and Z₁ are both propionyl.

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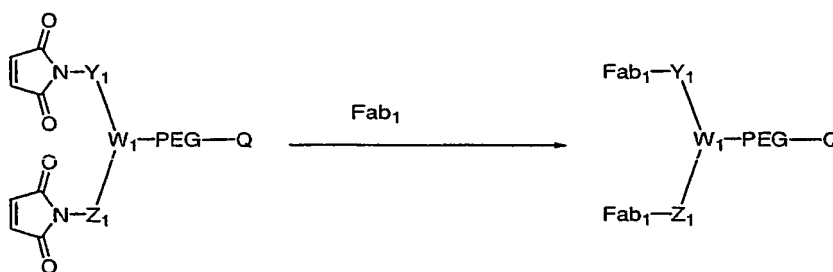
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In this and further examples, when the target binding moiety has an aldehyde or ketone functionality and the organic moiety contains a hydrazine functionality, then reductive alkylation may be used to form a covalent C-N bond. Another possibility is the reverse, where the target binding moiety contains a hydrazine functionality and the organic moiety contains an aldehyde or ketone, then reductive alkylation also leads to the formation of a covalent C-N bond. Alternatively, the target binding moiety can

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contain a single free -SH group and the organic moiety contains a bromoacetyl moiety, in which case, these groups spontaneously react (under appropriate pH control) to form a thioether bond. If, for example, the target binding moiety contains a hydrazine and the organic moiety contains a 1,3- di-carbonyl moiety or a 1,4-dicarbonyl moiety, then
 5 reaction of these functionalities would lead to stable 5- or 6-membered heterocyclic systems. The reverse configuration would also work: The target binding moiety could contain an azide and the organic moiety could contain a trivalent phosphorus moiety, giving spontaneous reaction for form a covalent phosphoramidate bond.

This type of bifunctional ΨAb construct has the advantage over a conventional
 10 Fab'₂ antibody fragment in that incorporation of the PEG can increase the molecular size of the construct to IgG size without the associated Fc activity.



Scheme 4

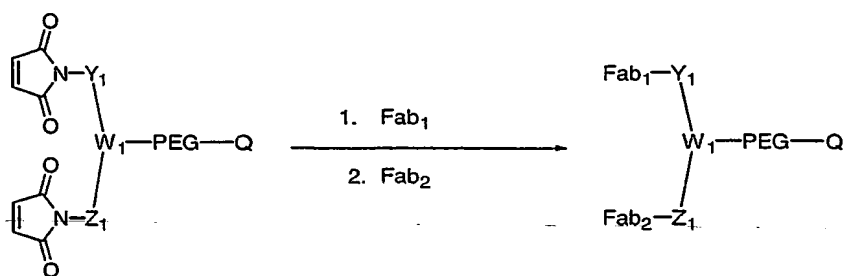
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Example 5.

Construct 5, shown in Scheme 5, has two different Fabs on the same end of a PEG, where Q can be an alkoxy group such as methoxyl or a compound selected from the structural categories of carbohydrates, saturated or unsaturated mono- or di-
 20 carboxylic acids, monoesters or amides of saturated or unsaturated di-carboxylic acids, higher alkoxy groups, lipids or other biologically compatible organic molecules. Y₁ and Z₁ are linkers or spacers between the maleimide moiety and the PEG, and can be the same or different. W is a trifunctional moiety such that one functionality can be attached to a PEG and the other two can be attached to the linkers Y₁ and Z₁. As an
 25 example, Q is methoxyl, PEG is NH₂-PEG, W₁ is Lysine and Y₁ and Z₁ are both propionyl. The synthesis of this type of construct is illustrated using sequential addition of the Fabs to a bis-maleimido-PEG, although other synthetic routes can be envisioned as well. This type of construct is well suited to a synthetic route in which the chemistry of attachment of the two Fabs is different, or the addition of one
 30 maleimide to the PEG is done after the addition of the first Fab. This type of

bifunctional Ψ Ab construct has the advantage over a conventional bifunctional antibody fragment in that incorporation of the PEG can increase the molecular size of the construct to IgG size without the associated Fc activity and additional activity can be imparted to these constructs by the Q group.

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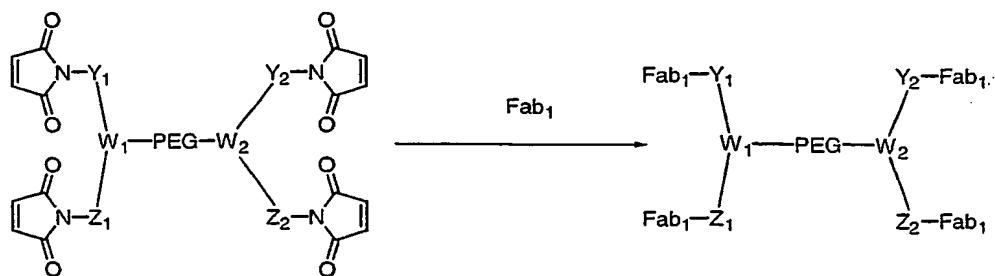


Scheme 5

Example 6.

Construct 6, shown in Scheme 6, has two different Fabs on each end of a PEG. Y_1 , Y_2 , Z_1 and Z_2 are linkers or spacers between the maleimide moiety and the PEG and can be the same or different. W_1 and W_2 are trifunctional moieties such that one functionality can be attached to a PEG and the other two can be attached to the linkers Y_1 , Y_2 , Z_1 and Z_2 . As an example, PEG is NH_2 -PEG, W_1 and W_2 are Lysine and Y_1 , Y_2 , Z_1 and Z_2 are propionyl. The synthesis of this type of construct is illustrated using addition of the Fabs to a bis-maleimido-PEG, although other synthetic routes can be envisioned as well. This type of tetravalent Ψ Ab construct has the advantage over a conventional antibody fragment in that incorporation of the PEG can increase the molecular size of the construct to IgG size without the associated Fc activity and the multiple binding capacity can increase avidity.

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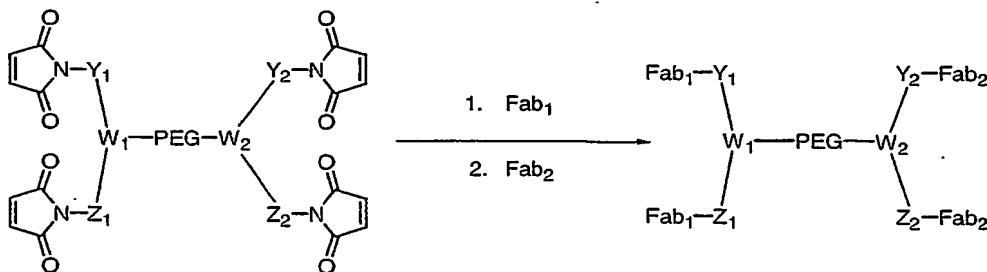


Scheme 6

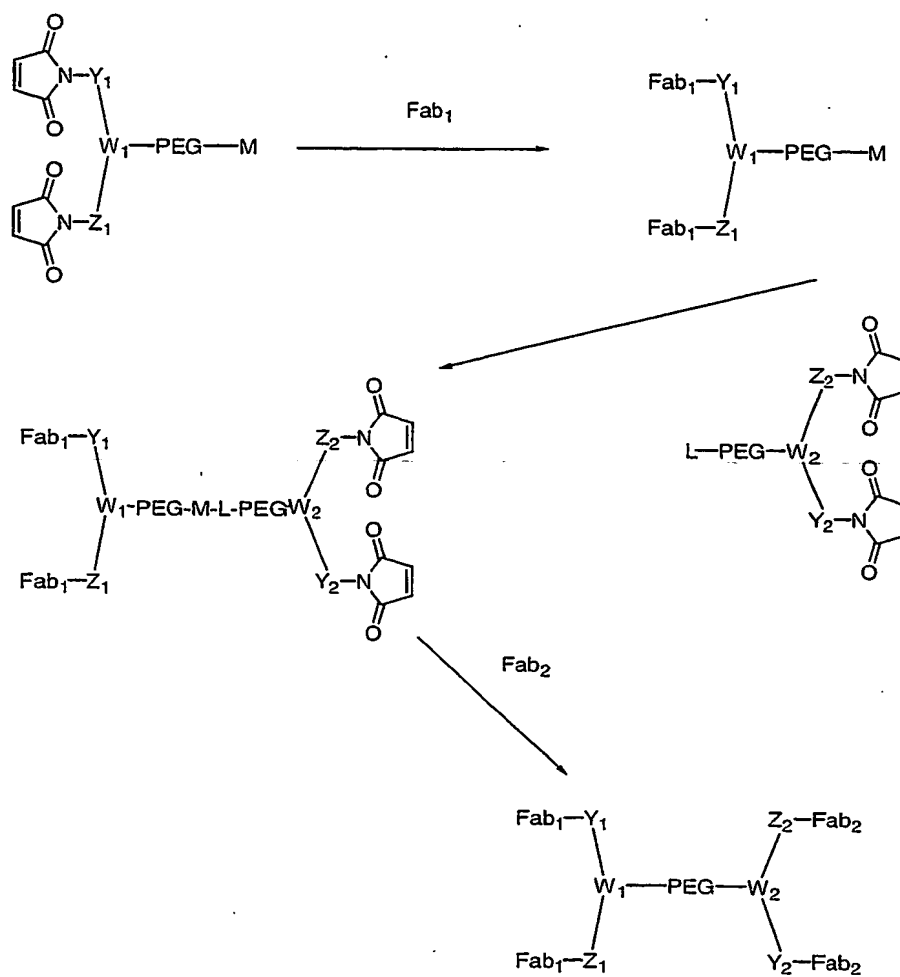
Example 7.

Construct 7, shown in Scheme 7, has two different sets of Fabs on opposite ends of a PEG. Y_1 , Y_2 , Z_1 and Z_2 are linkers or spacers between the maleimide moiety and the PEG and can be the same or different. W_1 and W_2 are trifunctional moieties such that one functionality can be attached to a PEG and the other two can be attached to the linkers Y_1 , Y_2 , Z_1 and Z_2 . As an example, PEG is NH_2 -PEG- NH_2 , W_1 and W_2 are Lysine and Y_1 , Y_2 , Z_1 and Z_2 are propionyl.

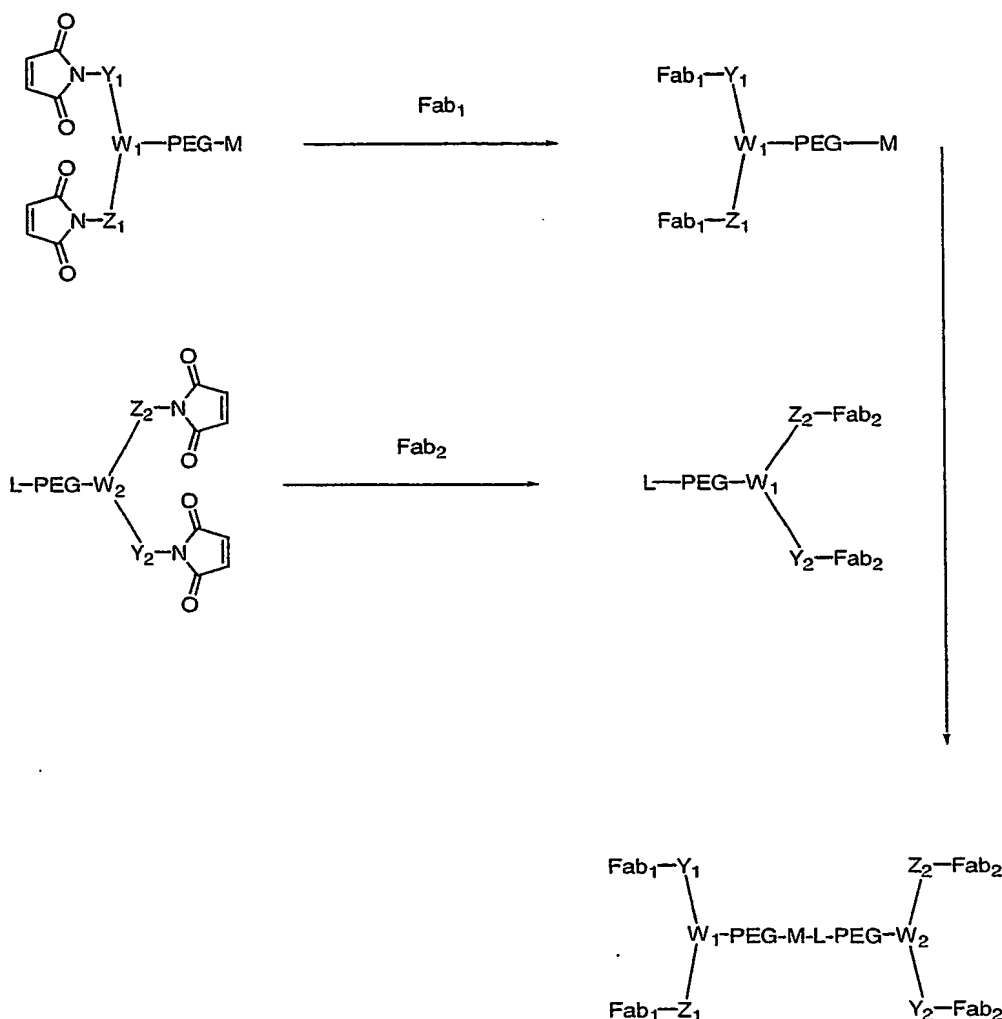
The synthesis of this type of construct is illustrated using sequential addition of the Fabs to a bis-maleimido-PEG in, although other synthetic routes can be envisioned as well. This type of tetravalent Ψ Ab construct has the advantage over a conventional antibody fragment in that incorporation of the PEG can increase the molecular size of the construct to IgG size without the associated Fc activity and the multiple binding capacity can increase avidity. Schemes 8 and 9 show two routes to these constructs, although other routes can be envisioned as well. L and M are groups that will react with groups at the ends of the PEG. For example L may be an active ester when the PEG moiety terminates in an amino group and would lead to the formation of an amide linkage or they may be hydrazides when the PEG moiety terminates in an aldehyde function and would lead to a hydrazide by way of reductive alkylation. Other groups may be envisioned as well. L and M may be identical or different depending on the specific assembly strategy. This type of bis- Ψ Ab construct has the advantage of being able to target two different antigens with IgG avidity in a single molecule.



Scheme 7



Scheme 8



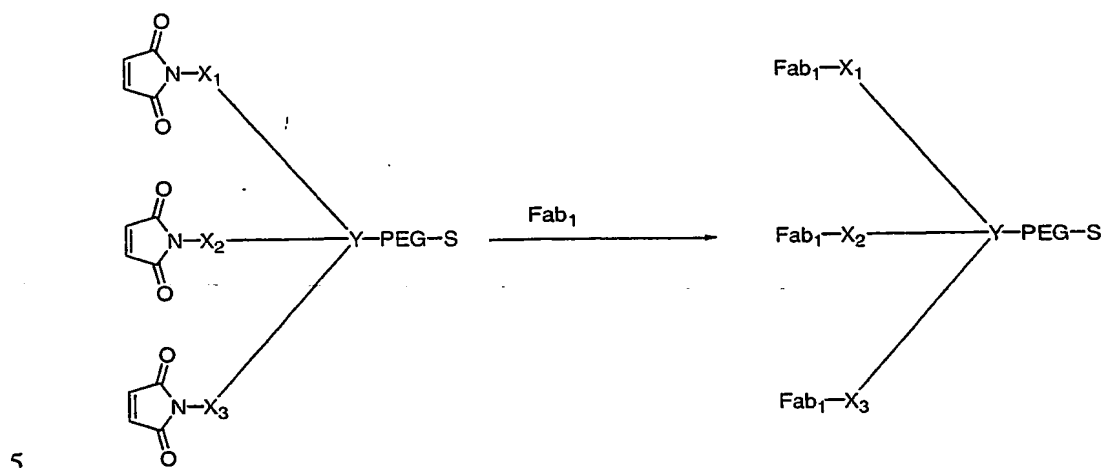
Scheme 9

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Example 8.

Construct 8, shown in Scheme 10, has three identical Fabs on the same end of a PEG where S can be H, an alkoxy group such as methoxyl or a compound selected from the structural categories of carbohydrates, saturated or unsaturated mono- or di-carboxylic acids, monoesters or amides of saturated or unsaturated di-carboxylic acids, higher alkoxy groups, lipids or other biologically compatible organic molecules. X₁, X₂ and X₃ are linkers or spacers between the maleimide moiety and the PEG and can be the same or different. Y is a trifunctional moiety such that one functionality can be attached to a PEG and the other two can be attached to the linkers X₁, X₂ and X₃. As an

example, S is methoxyl, PEG is NH_2 -PEG, Y is Lysyl-Lysine and X_1 , X_2 and X_3 are propionyl.

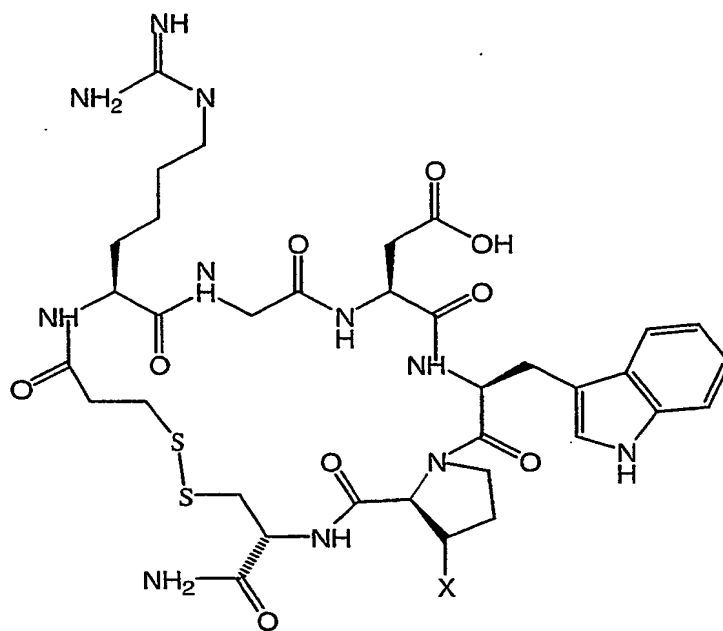


Scheme 10

In addition, one can readily envision higher order constructs with different numbers of identical or different Fabs attached to the ends of linear or branched PEGs or more complex structures involving multifunctional PEGs (e.g., NH_2 -PEG₁-NH-PEG₂-NH₂).

Example 9.

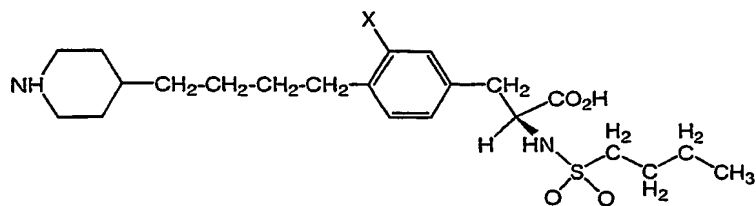
15 Examples of the types of structures that can be used as target binding moieties are REOPRO®-TC Fabs, where REOPRO® Fab is derived from the antibody c7E3 and TC represents the addition of threonyl-cysteine to the C-terminus of the heavy chain and the compound shown in Structure 1, capable of inhibiting platelet aggregation by binding to the GPIIb/IIIa receptor. Cysteines can be incorporated into other positions in a Fab as well. It need not be on the C-terminus. In this example, X is or contains a functional group capable of forming the Ψ Ab structure. Alternatively, X is hydrogen, and the carboxylic acid of cysteine forms an amide with an amino group that is attached to the organic moiety. Then, instead of NH_2 , as shown, it would be R-NH. The position of X is selected at any of those sites on the molecule at which substitution
25 allows the parent structure to retain some activity.



Structure 1

5 Example 10.

Another example of a structure that can be used for a target binding moiety is shown in Structure 2, a compound capable of inhibiting platelet aggregation by binding to the GPIIb/IIIa receptor, where X is or contains a functional group capable of forming the Ψ Ab structure. The position of X is selected at any of those aromatic sites on the molecule for which substitution will retain some activity of the parent structure, and is not limited to that position depicted in the drawing.



Structure 2

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Example 11.

Another example of a structure that can be used for a Fab is the peptide shown in Structure 3, a compound capable of binding to the erythropoietin receptor and stimulating erythropoiesis, where X is or contains a functional group capable of forming the Ψ Ab structure. One specific example is where X is an aldehyde containing moiety; however, other functional groups could be inserted as well. In the case where a cysteine is to be used to form the Ψ Ab structure, amino acids in the parent peptide could be substituted as well if they will not eliminate the activity of the parent structure. Preferably, attachment is at the amino- or carboxy-terminus of the molecule.

XGGTYS-cyclo(CHFGPLTWVC)-KPQGG

Structure 3

Example 12.

This example provides for a pseudo-antibody with the structure A-(PEG-Q)_n; wherein A is a Fab fragment, and Q is a fatty acid or lipid, and n is 1 or 2. Interestingly, the Fab-PEG-Q pseudo-antibody may have a greater circulating half-life compared to its counterpart Fab-PEG pseudo-antibody. In this example, Q is either diesteroylphosphatidyl-ethanolamine (DSPE) or palmitoyl (PAL). These pseudo-antibodies may be considered superior to unmodified Fabs, in that antigen-binding is retained while circulating half-life increases. Indeed, the increased circulating half-life may be advantageous even if antigen-binding activity is decreased by the addition of the organic moiety.

The organic moieties portions of these constructs may also be dimerized, such that n = 2. For example, the antibody fragment 7E3 Fab' was used to construct the pseudo-antibody 7E3 Fab'(PEG_{3,4k} - DSPE)₂ and the pseudo-antibody 7E3 Fab'(PEG_{3,4k} - PAL)₂ and the *in vitro* activities were compared with unmodified 7E3 Fab'. The activities of pseudo-antibodies and the unmodified Fab were similar, as indicated in Figure 1.

Additionally, 7E3 Fab' was used to construct the pseudo-antibodies 7E3 Fab'(PEG_{5k})₂ and 7E3 Fab'(PEG_{10k})₂ and the *in vitro* activities were compared with the unmodified antibody fragment ReoPro®. These constructs exhibited somewhat lower

in vitro activity than the unmodified antibody fragment, yet binding activity was clearly retained, as indicated in Figure 2.

For *in vivo* pharmacokinetic analysis, c7E3 Fab'(PEG_{3.4k}-DSPE)₂ and c7E3 Fab'(PEG_{5k}) were prepared, and given to mice in equimolar doses. The results are depicted in Figure 3. Although the c7E3 Fab'(PEG_{5k}) pseudo-antibody has a higher molecular weight and is larger than the c7E3 Fab'(PEG_{3.4k}-DSPE)₂ pseudo-antibody, it was cleared faster. The slower rate of clearance of the c7E3 Fab'(PEG_{3.4k}-DSPE)₂ pseudo-antibody construct may be contributed to the incorporation of the lipid moiety in the pseudo-antibody construct.

Other structures can be envisioned as well. Preferred structures are those that bind to a biological molecule to block binding to another biological molecule or bind to a biological molecule to initiate a biological event.

I Claim:

1. A pseudo-antibody comprising an organic moiety covalently coupled to three or more identical target-binding moieties, wherein said target-binding moieties are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule.
2. The pseudo-antibody of claim 1, wherein said pseudo-antibody exhibits increased avidity compared to the unmodified target-binding moiety from which it is derived.
3. The pseudo-antibody of claim 1, wherein said organic moiety is selected from the group consisting of a hydrophilic polymeric group, a fatty acid group, a fatty acid ester group, a simple carbohydrate, a complex carbohydrate, a lipid, and a phospholipid.
4. The pseudo-antibody of claim 3, wherein said organic moiety is a hydrophilic polymeric group.
5. The pseudo-antibody of claim 4, wherein said hydrophilic polymeric group is present on a polyethylene glycol (PEG) molecule.
6. The pseudo-antibody of claim 5, wherein said PEG molecule of sufficient size to extend the *in vivo* half-life of an unmodified target-binding moiety.
7. The pseudo-antibody of claim 1, wherein said target-binding moiety inhibits binding of fibrinogen to GPIIb/IIIa.
8. The pseudo-antibody of claim 1, wherein said target-binding moiety is a protein selected from the group consisting of an antibody, a cytokine, a growth factor, a cell cycle protein, a blood protein, an integrin, a receptor, a neurotransmitter, an antigen, an anti-microbial agent, and any functional or structural equivalent of any of the foregoing.

9. The pseudo-antibody of claim 1, wherein said target-binding moiety is a protein that is a receptor or a functional portion of a receptor for a molecule selected from the group consisting of an antibody, a cytokine, a growth factor, a cell cycle protein, a blood protein, an integrin, a neurotransmitter, an antigen, an anti-microbial agent, and any functional or structural equivalent of any of the foregoing.
10. The pseudo-antibody of claims 8, wherein said target-binding moiety is a Fab.
11. The pseudo-antibody of claim 10, wherein the binding of said Fab to GPIIb/IIIa is competitively inhibited by 7E3.
12. The pseudo-antibody of claim 11, wherein said Fab is selected from the group consisting of 7E3, antigen-binding fragments of 7E3, chimerized 7E3, antigen-binding fragments of chimeric 7E3, humanized 7E3, and antigen-binding fragments of humanized 7E3.
13. The pseudo-antibody of claim 11, wherein said Fab has an increased *in vivo* serum half-life, compared to an unmodified antibody or unmodified Fab that is competitively inhibited by 7E3.
14. The pseudo-antibody of claim 4, wherein said hydrophilic polymeric group is selected from the group consisting of, linear or branched polyalkane glycol chains, carbohydrate chains, amino acid chains and polyvinyl pyrrolidone chains; wherein said hydrophilic polymeric group has a molecular weight of about 800 Daltons to about 120,000 Daltons.
15. The pseudo-antibody of claim 14, wherein said hydrophilic polymeric group is a linear or branched polyalkane glycol chain with a molecular weight greater than about 2,000 Daltons.
16. A pseudo-antibody comprising an organic moiety covalently coupled to two or more different target-binding moieties, wherein said target-binding moieties are

selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule.

17. The pseudo-antibody of claim 16, wherein said pseudo-antibody exhibits increased avidity compared to the unmodified target-binding moiety from which it is derived.

18. The pseudo-antibody of claim 16 wherein said organic moiety is selected from the group consisting of a hydrophilic polymeric group, a fatty acid group, a fatty acid ester group, a simple carbohydrate, a complex carbohydrate, a lipid, and a phospholipid.

19. The pseudo-antibody of claim 18, wherein said organic moiety is a hydrophilic polymeric group.

20. The pseudo-antibody of claim 19, wherein said hydrophilic polymeric group is present on a polyethylene glycol (PEG) molecule.

21. The pseudo-antibody of claim 20, wherein said PEG molecule of sufficient size to extend the *in vivo* half life of said unmodified target-binding moiety.

22. The pseudo-antibody of claim 16, wherein said target-binding moiety inhibits binding of fibrinogen to GPIIb/IIIa.

23. The pseudo-antibody of claim 16, wherein said target-binding moiety is a protein selected from the group consisting of an antibody, a cytokine, a growth factor, a cell cycle protein, a blood protein, an integrin, a receptor, a neurotransmitter, an antigen, an anti-microbial agent, and any functional or structural equivalent of any of the foregoing.

24. The pseudo-antibody of claim 16, wherein said target-binding moiety is a protein that is a receptor or a functional portion of a receptor for a molecule selected from the group consisting of an antibody, a cytokine, a growth factor, a cell cycle protein, a blood protein, an integrin, a neurotransmitter, an antigen, an anti-microbial agent, and any functional or structural equivalent of any of the foregoing.

25. The pseudo-antibody of claims 23, wherein said target-binding moiety is a Fab.

26. The pseudo-antibody of claim 25, wherein the binding of said Fab to GPIIb/IIIa is competitively inhibited by 7E3.

27. The pseudo-antibody of claim 26, wherein said Fab is selected from the group consisting of 7E3, antigen-binding fragments of 7E3, chimeric 7E3, an antigen-binding fragment of chimeric 7E3, humanized 7E3, and antigen-binding fragments of humanized 7E3.

28. The pseudo-antibody of claim 26, wherein said Fab has an increased *in vivo* serum half-life, compared to an unmodified antibody or unmodified Fab that is competitively inhibited by 7E3.

29. The pseudo-antibody of claim 18, wherein said hydrophilic polymeric group is selected from the group consisting of, linear or branched polyalkane glycol chains, carbohydrate chains, amino acid chains and polyvinyl pyrrolidone chains; wherein said hydrophilic polymeric group has a molecular weight of about 800 Daltons to about 120,000 Daltons.

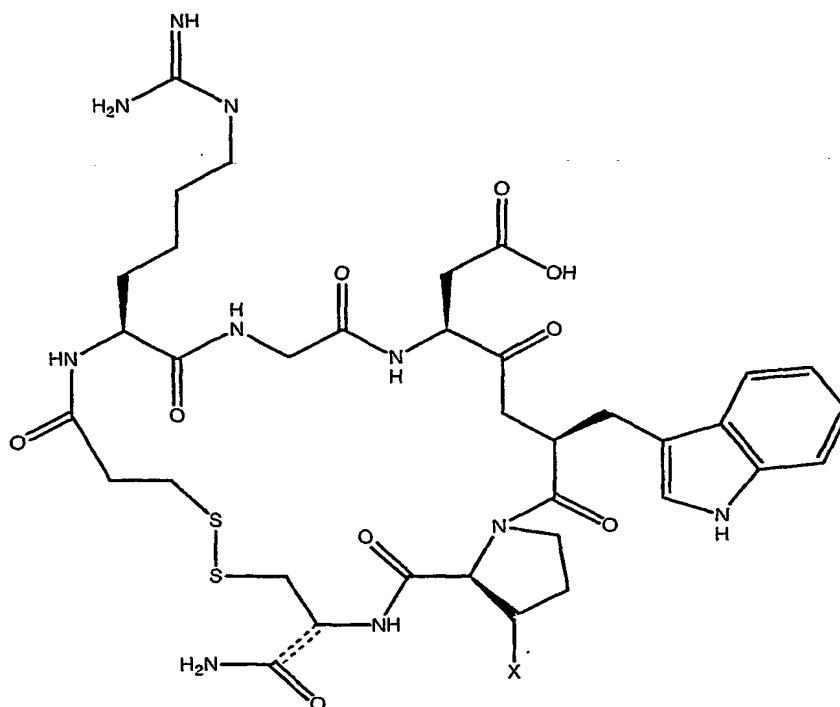
30. The pseudo-antibody of claim 29, wherein said hydrophilic polymeric group is a linear or branched polyalkane glycol chain with a molecular weight greater than about 2,000 Daltons.

31. A pharmaceutical composition comprising a multivalent pseudo-antibody comprising two or more target-binding moieties covalently coupled to a functional molecule.

32. The pharmaceutical composition of claim 31, wherein said functional molecule is a GPIIb/IIIa antagonist.

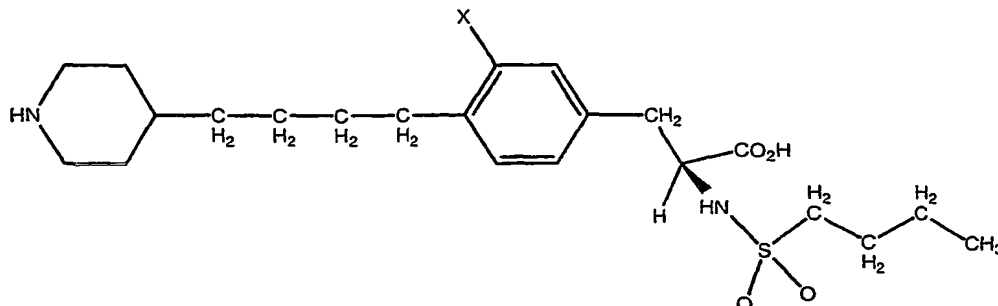
33. The pharmaceutical composition of claim 31, wherein said target-binding moiety is a *GI*Ib/IIIa antagonist.

34. The pharmaceutical composition of claim 32, wherein said pseudo-antibody comprises the following structure:



wherein X is or contains a functional group capable of forming the pseudo-antibody structure.

35. The pharmaceutical composition of claim 31, wherein said pseudo-antibody comprises the following structure:



wherein X is or contains a functional group capable of forming the pseudo-antibody structure.

36. A pharmaceutical composition comprising a dimerized peptidomimetic that exhibits enhanced binding to an EPO receptor as compared to its monomeric peptidomimetic.

37. The pharmaceutical composition of claim 36, wherein the dimerized peptidomimetic has the structure:



wherein X is hydrazine.

38. The pseudo-antibody of claim 1, further comprising a linker molecule between said antigen-binding-fragment and said organic moiety.

39. The pseudo antibody of claim 16, further comprising a linker molecule between said antigen-binding-fragment and said organic moiety.

40. The pseudo-antibody of claim 1, further comprising an additional functional molecule.

41. The pseudo-antibody of claim 16, further comprising an additional functional molecule.

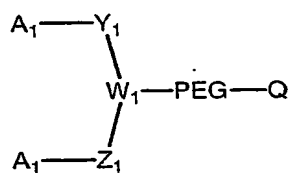
42. A pseudo-antibody comprising the structure $A_1-X_1\text{-PEG-}X_2\text{-}A_2$, wherein A_1 and A_2 are different target-binding moieties each selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule, wherein X_1 and X_2 are optional linkers between the PEG and the A moieties.

43. The pseudo-antibody of claim 42, wherein said linkers are structurally identical.

44. The pseudo-antibody of claim 42, wherein said linkers structurally unique.

45. The pseudo-antibody of claim 42, wherein said either or both of A₁ or A₂ is a Fab.

46. A pseudo-antibody having the following structure:



wherein A₁ is selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule;

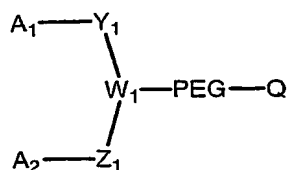
wherein Q can be an alkoxy group, such as methoxyl, or a compound selected from the group of structural categories consisting of a carbohydrate, a saturated or unsaturated mono- or di-carboxylic acid, a monoester or amide of a saturated or unsaturated di-carboxylic acid, a higher alkoxy group, a lipid, or other biologically compatible organic molecule;

wherein Y₁ and Z₁ are linkers or spacers between the maleimide moiety and the PEG and can be the same or different; and

wherein W₁ is a trifunctional moiety such that one functionality can be attached to a PEG and the other two can be attached to the linkers Y₁ and Z₁ or directly to A₁ and A₂.

47. The pseudo-antibody of claim 46, in which Q is methoxyl, PEG is NH₂-PEG, W₁ is Lysine, and Y₁ and Z₁ are both propionyl.

48. A pseudo-antibody having the following structure:



wherein A_1 and A_2 are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule, with the proviso that A_1 and A_2 are not identical;

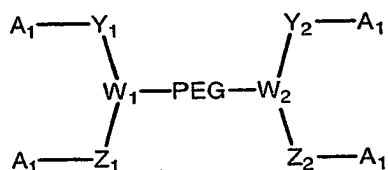
wherein Q can be an alkoxy group, such as methoxyl, or a compound selected from the group of structural categories consisting of a carbohydrate, a saturated or unsaturated mono- or di-carboxylic acid, a monoester or amide of a saturated or unsaturated di-carboxylic acid, a higher alkoxy group, a lipid, or other biologically compatible organic molecule;

wherein Y_1 and Z_1 are optional linkers or spacers between the maleimide moiety and the PEG; and

wherein W_1 is a trifunctional moiety such that one functionality can be attached to a PEG and the other two can be attached either to the linkers Y_1 and Z_1 , or directly to A_1 and A_2 .

49. The pseudo-antibody of claim 48, wherein Q is methoxyl, PEG is NH_2 -PEG, W_1 is Lysine and Y_1 and Z_1 are both propionyl.

50. A pseudo-antibody comprising the following structure:



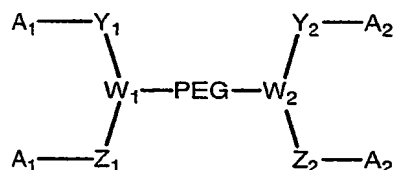
wherein A_1 is selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule;

wherein Y_1 , Y_2 , Z_1 and Z_2 are optional linkers or spacers between the maleimide moiety and the PEG; and

wherein W_1 and W_2 are trifunctional moieties such that one functionality can be attached to a PEG and the other two can be attached either to the linkers Y_1 , Y_2 , Z_1 and Z_2 , or directly to the A_1 moiety.

51. The pseudo-antibody of claim 50, wherein PEG is $\text{NH}_2\text{-PEG}$, W_1 and W_2 are Lysine and Y_1 , Y_2 , Z_1 and Z_2 are propionyl.

52. A pseudo-antibody comprising the following structure:



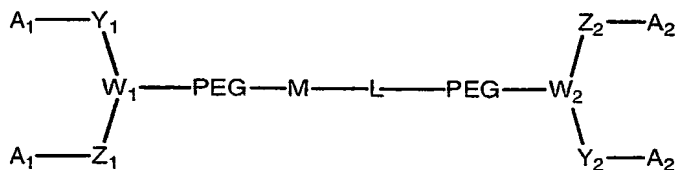
wherein A_1 and A_2 are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule, with the proviso that A_1 and A_2 are not identical;

wherein Y_1 , Y_2 , Z_1 and Z_2 are optional linkers or spacers between the maleimide moiety and the PEG and can be the same or different; and

wherein W_1 and W_2 are trifunctional moieties such that one functionality can be attached to a PEG and the other two can be attached either to the linkers Y_1 , Y_2 , Z_1 and Z_2 , or directly to the A_1 moiety.

53. The pseudo-antibody of claim 52, wherein PEG is $\text{NH}_2\text{-PEG-NH}_2$, W_1 and W_2 are Lysine and Y_1 , Y_2 , Z_1 and Z_2 are propionyl.

54. A pseudo-antibody comprising the following structure:



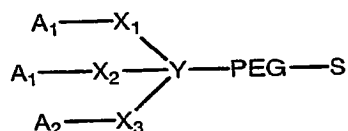
wherein A_1 and A_2 may be identical or different, each selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule;

wherein Y_1 , Y_2 , Z_1 and Z_2 are optional linkers or spacers between the maleimide moiety and the PEG and can be the same or different;

wherein W_1 and W_2 are trifunctional moieties such that one functionality can be attached to a PEG and the other two can be attached either to the linkers Y_1 , Y_2 , Z_1 and Z_2 , or directly to the A_1 and A_2 fragments; and

wherein M and L are identical or different, each selected from the group consisting of an amide, an ester, a thioamide, a thioester, a disulfide, and another covalent bond formed by two individual, compatible functional groups.

55. A pseudo-antibody comprising the following structure:



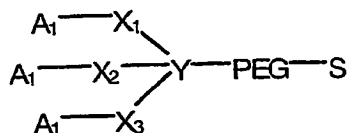
wherein A_1 and A_2 are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule, with the proviso that A_1 and A_2 are not identical;

wherein S is a hydrogen, an alkoxy group, such as methoxyl, or a compound selected from the structural categories consisting of a carbohydrate, a saturated or unsaturated mono- or di-carboxylic acid, a monoester or amide of a saturated or unsaturated di-carboxylic acid, a higher alkoxy group, a lipid, and an other biologically compatible organic molecules;

wherein X_1 , X_2 and X_3 are linkers or spacers between the maleimide moiety and the PEG and can be the same or different; and

wherein Y is a multifunctional moiety such that one functionality can be attached to a PEG and the other three can be attached to the linkers X_1 , X_2 and X_3 .

56. The pseudo-antibody comprising the following structure:



wherein A_1 is selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological

molecule; S is methoxyl; PEG is NH₂-PEG; Y is Lysyl-Lysine; and X₁, X₂ and X₃ are propionyl.

57. A pseudo-antibody comprising the following structure: A₁-(PEG-Q)_n; wherein A₁ is selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule; Q is selected from the group consisting of a fatty acid and a lipid; n is 1 or more, and wherein said A₁-(PEG-Q)_n pseudo-antibody has a greater circulating half-life compared to its counterpart A₁-(PEG)_n.

58. The pseudo-antibody of claim 57, in which Q is diesteroylphosphatidylethanolamine.

59. The pseudo-antibody of claim 57, in which Q is palmitoyl.

60. A molecule that binds to a primary biological molecule, having at least one or more of the following characteristics selected from the groups consisting of:

- multivalent structure with enhanced avidity;
- increased molecular size with extended circulating half-life;
- specific binding to multiple compounds by a single molecule; and
- incorporation of carriers such as lipids, fatty acids, carbohydrates and steroids, that can bind to molecules other than the primary biological molecules and affect distribution to specific locations.

61. A method of inhibiting stenosis and/or restenosis following a vascular intervention procedure in a human comprising administering to said human an effective amount of a composition comprising the pseudo-antibody of claim 1 or claim 16.

62. A method of preventing ischemia in a human comprising administering to said human an effective amount of the pseudo-antibody of claim 1 or claim 16.

63. A method of inhibiting the growth and/or metastasis of a tumor in a human comprising administering to said human an effective amount of the pseudo-antibody of claim 1 or claim 16.

64. A method of inhibiting a process mediated by the binding of a ligand to one of the group consisting of GPIIb/IIIa, $\alpha_v\beta_3$ and both GPIIb/IIIa, $\alpha_v\beta_3$, expressed on the plasma membrane of a cell in a human, comprising administering to said human an effective amount of the pseudo-antibody of claim 1 or claim 16.

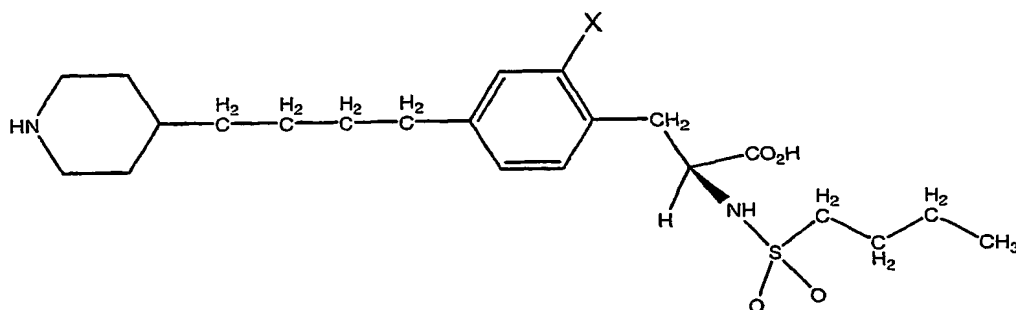
65. A method of inhibiting angiogenesis in a human comprising administering to said human an effective amount of the pseudo-antibody of claim 1 or claim 16.

66. The pharmaceutical composition of claim 36, wherein the dimerized peptidomimetic has the structure:



wherein R is an organic moiety, and the linkage between the carboxylic acid of glycine and R is an amide bond.

67. The pharmaceutical composition of claim 31, wherein said pseudo-antibody comprises the following structure, wherein X is or contains a functional group capable of forming the pseudo-antibody:



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(71) Applicant (*for all designated States except US*): CENTO-COR, INC. [US/US]; 200 Great Valley Parkway, Malvern, PA 19355 (US).

Published:
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(72) Inventor; and

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(88) Date of publication of the international search report:
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(74) Agents: JOHNSON, Philip, S. et al.; One Johnson & Johnson Plaza, New Brunswick, NJ 08933 (US).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PSEUDO-ANTIBODY CONSTRUCTS

(57) Abstract: This invention relates to novel pharmaceutically useful compositions that bind to a biological molecule, having improved circulatory half-life, increased avidity, increased affinity, or multifunctionality, and methods of use thereof. The present invention provides a pseudo-antibody comprising an organic moiety covalently coupled to at least two target-binding moieties, wherein the target-binding moieties are selected from the group consisting of a protein, a peptide, a peptidomimetic, and a non-peptide molecule that binds to a specific targeted biological molecule. The pseudo-antibody of the present invention may affect a specific ligand *in vitro*, *in situ* and/or *in vivo*. The pseudo-antibodies of the present invention can be used to measure or effect in a cell, tissue, organ or animal (including humans), to diagnose, monitor, modulate, treat, alleviate, help prevent the incidence of, or reduce the symptoms of, at least one condition.



WO 2003/049684 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/38839

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/395; C07K 16/00

US CL : 530/387.1, 387.3, 388.85, 391.1, 391.3; 424/130.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.1, 387.3, 388.85, 391.1, 391.3; 424/130.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,837,242 A (HOLLIGER ET AL) 17 November 1998 (17/11/98), see entire document, especially abstract, column 20, 23.	1-8, 10-14, 38, 40
Y	KITAMURA et al. Polyethylene glycol modification of monoclonal antibody A7 enhances its tumor localization. Biochem Biophys. Research Comm. 28 September 1990, Vol.171, pages 1387-94, especially abstract and page 1188.	1-8, 10-14, 38, 40
Y	US 5,976,532 A (COLLER ET AL) 02 November 1999 (02/11/99), see entire document, especially column 1-2.	7, 11-13

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 August 2003 (18.08.2003)

Date of mailing of the international search report

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/38839

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-8, 10-14, 38, 40 in part

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-8, 10-14, 38, 40 in part, drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is an antibody.

Group II, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is a cytokine.

Group III, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is a growth factor.

Group IV, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is a cell cycle protein.

Group V, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is a blood protein.

Group VI, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is an integrin.

Group VII, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is a receptor.

Group VIII, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is a neurotransmitter.

Group IX, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is an antigen.

Group X, claim(s) 1-6, 8-9, 14-15, 38, 40, in part drawn to a pseudo-antibody with three or more target binding moieties wherein the target-binding moiety is an anti-microbial agent.

Group XI, claim(s) 16-28, 39, 41-45 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is an antibody.

Group XII, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is a cytokine.

Group XIII, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is a growth factor.

Group XIV, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is a cell cycle protein.

Group XV, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is a blood protein.

Group XVI, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is an integrin.

Group XVII, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is a receptor.

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US02/38839

Group XVIII, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is a neurotransmitter.

Group XIX, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is an antigen.

Group XX, claim(s) 16-31, 39, 41-44 in part drawn to a pseudo-antibody with two or more target binding moieties wherein the target-binding moiety is an anti-microbial agent.

Group XXI, claim(s) 31-34 in part and claim 67 drawn to a pharmaceutical composition of the structure in claim 34.

Group XXII, claim(s) 31 and 35 in part drawn to a pharmaceutical composition of the structure in claim 35.

Group XXIII, claim(s) 36-37 and 66 drawn to a pharmaceutical composition of the structure in claim 37.

Group XXIV, claim(s) 46-47 drawn to a pseudo antibody with the structure in claim 46-47.

Group XXV, claim(s) 48-49 drawn to a pseudo antibody with the structure in claim 48-49.

Group XXVI, claim(s) 50-51 drawn to a pseudo antibody with the structure in claim 50-51.

Group XXVII, claim(s) 52-53 drawn to a pseudo antibody with the structure in claim 52-53.

Group XXVIII, claim(s) 54 drawn to a pseudo antibody with the structure in claim 54.

Group XXIX, claim(s) 55 drawn to a pseudo antibody with the structure in claim 55.

Group XXX, claim(s) 56 drawn to a pseudo antibody with the structure in claim 56.

Group XXXI, claim(s) 57-59 drawn to a pseudo antibody with the structure in claim 57-59.

Group XXXII, claim(s) 60 drawn to a molecule.

Group XXXIII, claim(s) 61 drawn to a method of inhibiting stenosis.

Group XXXIV, claim(s) 62 drawn to a method of preventing ischemia.

Group XXXV, claim(s) 63 drawn to a method of inhibiting the growth of a tumor.

Group XXXVI, claim(s) 64 drawn to a method of inhibiting a process.

Group XXXVII, claim(s) 65 drawn to a method of inhibiting angiogenesis.

The inventions listed as Groups I-XXXVII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The technical feature linking Groups I-XXXVII appears to be a targeting binding moiety of two or more covalently coupled to an organic moiety. Holliger et al (US Patent 5,837,242) who teach diabodies and triabodies in view of Kitamura et al (BBRC 171:1387-1394, 1990) who teach coupling of PEG to antibody fragments renders the technical feature not special. Therefore, the technical feature linking the inventions does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

INTERNATIONAL SEARCH REPORT

PCT/US02/38839

Continuation of B. FIELDS SEARCHED Item 3:

CAPLUS, MEDLINE, BIOSIS, WEST

Search terms: multivalent antibody, PEG, triabody, diabody, GPIIb/IIIa, conjugate, 7E3, chimeric, 800kDa, 1200kDa, label.

Form PCT/ISA/210 (second sheet) (July 1998)

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